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PHYSIOLOGICAL CONSEQUENCES OF LONG DURATION FLIGHT IN THE MIGRATORY GRASSHOPPER, *MELANOPLUS SANGUINIPES* FABRICIUS

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by

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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

-J. R. R. Tolkien-

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PHYSIOLOGICAL CONSEQUENCES OF LONG DURATION FLIGHT IN THE MIGRATORY GRASSHOPPER, *MELANOPLUS SANGUINIPES* FABRICIUS

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This study sought to examine the physiological correlates of migratory flight performance the North American migratory grasshopper *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) with a focus on mechanisms of resource allocation, the dynamics of hemolymph proteins, their interface with immune function, and the mechanism of flight-enhanced oogenesis. The performance of long duration flights has been shown previously to be of reproductive benefit to females who make them. Examination of possible mechanisms of resource compensation for the costs of flight showed no significant increase in either feeding, mating or digestion in females who performed long duration flight. A comparison of two populations of *M. sanguinipes* from Arizona and Colorado showed significant variation in body size,

diapause regulation as well as internal and external morphology. The two populations did not differ in taxonomic characters or in short sequences of genomic and mitochondrial DNA. The follicle cell epithelium of ovaries from *M. sanguinipes* was examined for its relationship to juvenile hormone III (JH III). JH III induces patency *in vitro* in intercellular spaces of *M. sanguinipes* follicular epithelium as well as the characteristic apical endocytosis at the follicle cell oocyte interface. Exogenous JH III treatment of females on day 7 *in lieu* of flight reduced the threshold for induction of patency to 10^{-7} M JH III from 10^{-5} M JH III. These results indicate that JH III can act as a prime to the pump of oogenesis. An HPLC/LC-MS peptidomic survey of the hemolymph of *M. sanguinipes* following flight performance showed the presence of and changes in serine protease inhibitors. These peptides regulate numerous protease cascades involved in reproduction and immunity which suggested that flight might have a more broad impact than previously thought. Males who performed these flights showed a higher probability of surviving a bacterial challenge. The duration of flight performance was positively correlated in males with increases in titers of the hemolymph lipoproteins apolipophorin I and hexemerin. The exchangeable apolipophorin III showed no variation in correlation with flight. Females were not affected by flight performance in terms of hemolymph protein titers or the probability of surviving a bacterial challenge. These results suggest that the lipid transport system plays an important role in the immune response of this insect.

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CHAPTER 1

General Introduction

Migratory insects often have a serious impact on agriculture. The most important of these in terms of economic damage are the gregarious swarms of the African locusts *Locusta migratoria* and *Schistocerca gregaria*, whose movements and behavior can lead to massive crop loss (Johnson, 1969; Van Huis et al., 2007). In 2004, plague locust damages and control costs were estimated at 3 billion $US (Food and Agriculture Organization of the United Nations, 2004). The migratory grasshopper *Melanoplus sanguinipes* is the most destructive pest species of rangeland vegetation in North America (Hewitt, 1977). Outbreaks of *M. sanguinipes* have occurred several times this century (Parker *et al.*, 1955) and migratory flights are common even in non-outbreak years (McAnelly, 1985). Unlike the phase polymorphic locust *Schistocerca gregaria,* the probability of individual *M. sanguinipes* making long migratory flights is somewhat independent of cues such as density or photoperiod. In this species there is a considerable degree of heritable, intra- and interpopulation variability in migratory behavior (McAnelly, 1984; McAnelly & Rankin, 1986a; Kent and Rankin, 2001). This study sought to examine the physiological correlates of migratory flight performance in *M. sanguinipes* with a focus on mechanisms of resource

allocation, the dynamics of hemolymph proteins, their interface with immune function, and the mechanism of flight-enhanced oogenesis.

Long Duration Flight Performance in Insects

Migration is an important component of the life history of numerous insect species. It can 1: increase the foraging and reproductive range of an insect, 2: increase the probability of aggregation and encounter of conspecifics, 3: provide escape from potential predation or deteriorating environmental conditions such as changes in climate and food supply and/or 4: enable colonization of new habitat patches (Dingle, 1996).

Insect migrants like locusts often ride weather fronts and control flight direction by varying their altitude like a human balloonist (Kennedy, 1961; Johnson, 1965; Srygley and Dudley, 2008). Over the last five decades an operational definition of migratory behavior has been established through the careful behavioral analyses of Kennedy, Dingle and their students (Kennedy, 1961, 1965; Dingle, 1996). A fundamental distinction between migratory flight and appetitive flight behavior is that during migratory flight the insect is not distracted by stimuli that would otherwise elicit settling behavior such as cues for feeding, mating, or oviposition sites (Kennedy, 1961). Kennedy analyzed the migratory behavior of aphids in a wind tunnel that provided migratory cues (light, sustained wind) and also allowed the presentation of cues associated with host plants and conspecifics. Since aphids typically take off by flying towards sunlight,

they could be induced to fly upwind or against the wind towards a bright light and could be held in place in the wind chamber by variable wind pressure while being presented with appetitive cues. In these experiments Kennedy characterized migratory flight behavior and showed, among other things, that migratory behavior typically occurs in the pre-reproductive adult (Kennedy & Booth, 1963). The criteria of duration, persistence, directionality, and undistractability of flight have been used as characters of migratory flight in many subsequent studies, based on Kennedy's elegant behavioral analyses (McAnelly, 1986a; Rankin & Singer, 1986; Rankin, 1989; Dingle, 1996; Kent, 1999).

Dingle and colleagues showed in *Oncopeltus fasciatus* that migratory individuals would fly for several hours in repeated flight tests while non-migratory conspecifics typically made only very short flights of a few minutes or less (reviewed in Dingle, 1972). Thus Dingle concluded if a milkweed bug flew for 30 minutes, it would fly much longer and be considered a migrant. This dichotomy of flight behavior has been observed in a number of other species and allows for more abbreviated flight tests to identify migrants in a population. As with other migratory insects described above, individual *M. sanguinipes* tend to fly for only a few moments or for hours (McAnelly, 1985). A sixty-minute flight performance can reliably distinguish migrants and non-migrants in a population (McAnelly, 1984; Burchsted, 1990; Kent, 1990; Min, 2003). This one-hour rule developed by McAnelly (1984) is used to identify migrants by their completion of one hour of flight. In our experiments the individuals identified as migrants are then used as a

control group for comparison with migrants that are allowed to fly until voluntary cessation.

The migratory status of individual insects may be considered on several levels. Depending on the species, variation in the capacity for migration may include intraspecific wing length polymorphisms, developmental plasticity and/or histolysis of the flight muscles in non-migrants; or even variation in the amount and mobilization of energy reserves (Zera and Brink, 2000; Zera and Larson, 2001; Zhao and Zera, 2001).

The relationship between flight and reproduction in insects is complex. Because migratory flight and reproduction are metabolically expensive and draw on the same reserves, the two are often viewed as alternate physiological states. Indeed, early work by Johnson, Kennedy, and Dingle (Johnson, 1969; Kennedy, 1961; Dingle, 1966) emphasized the pre-reproductive timing of migratory behavior in many species. Rankin (Rankin and Riddiford, 1977; 1978; Rankin 1980) investigated the physiological relationship between migratory behavior and reproductive development in a typical insect migrant, the milkweed bug *Oncopeltus fasciatus*, and showed that although this species displays a typical oogenesis-flight syndrome, both migration and reproduction are actually stimulated by the same hormone (juvenile hormone) and are a part of a coordinated life history strategy that emerges in response to appropriate environmental cues.

In many species the onset of ovarian development is accompanied by the loss of flight propensity or even capacity. In wing-dimorphic species, short-winged or wingless individuals often exhibit increased fecundity (Mole & Zera, 1993). In *M. sanguinipes* performance of long duration flight by females accelerates the time to first oviposition and enhances overall reproductive output (McAnelly & Rankin, 1986b; Rankin & Burchsted, 1992; Min et al., 2004).

The physiological correlates of migratory flight have typically been studied primarily in females (Dingle, 1996). How the capacity for or performance of migration influences male physiology should also be a fruitful line of inquiry, particularly in a species such as *M. sanguinipes* where the resource contribution of males to reproduction is substantial (Friedal and Guilloit, 1977). Understanding the impact of migration on both male and female insects is an important step in establishing a more integrated picture of insect migratory physiology and the evolution of migration as a life history strategy.

Oogenesis

Female insects possess a pair of bilateral ovaries connected to a single central common oviduct by a lateral oviduct. Each ovary is composed of a number of ovarioles or egg tubes that descend from a terminal filament that functions as an anchor for the ovary in the hemocoel and attaches to the abdominal wall at its anterior end. Each of the ovarioles within the ovary produces a series of oocytes, the most mature being the most proximal to the

oviduct (Stys and Blinksi, 1990). Three major types of ovaries can be distinguished; panositic, telotrophic meroistic, and polytrophic meroistic (Fig 1.1) (Heming, 2003). Panositic ovaries are thought to be the least derived evolutionarily with the maturing oocytes linearly arranged along the ovariole and invested with a sheath of somatic cells referred to as the follicular epithelium.

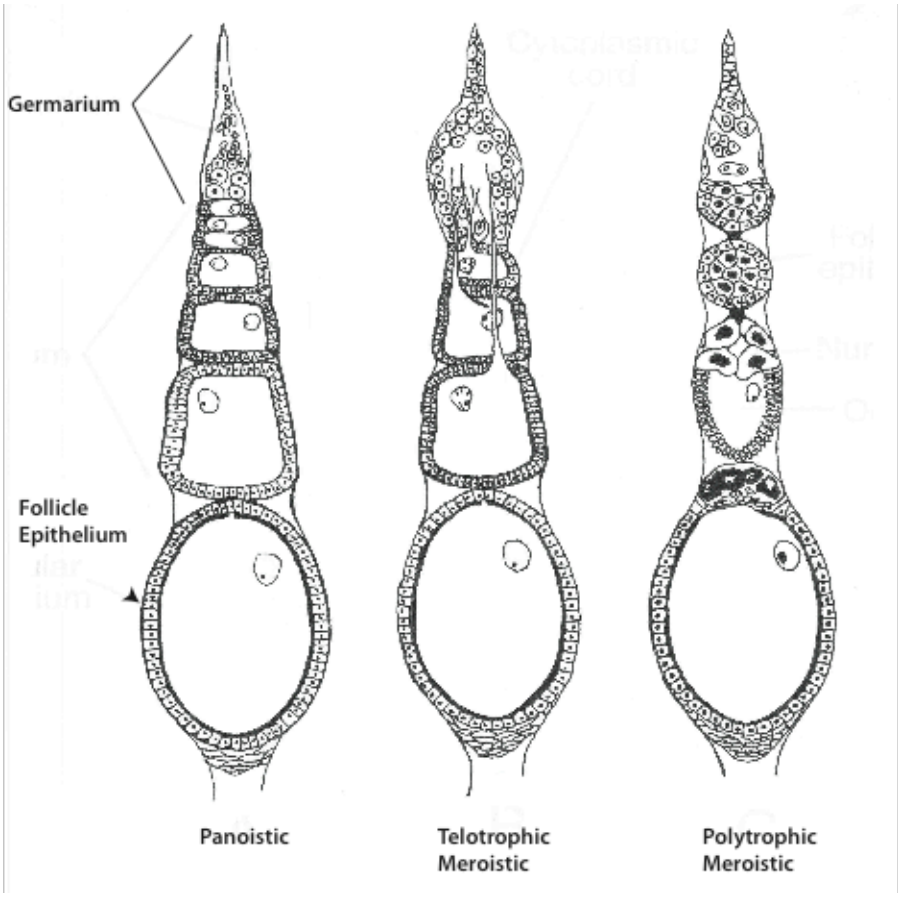


Figure 1.1 The three main types of insect ovariole.
Panositic ovaries are the simplest with the oocyte descending alone to its terminal position, telotrophic meroistic ovarioles in which the oocytes descent is trailed by a nutritive cord and polytrophic meroistic ovarioles in which the oocytes descent is accompanied by a set of sibling nursecells. Figure adapted from Schwalm (1988).

This type of ovary is found in the insect orders *Orthoptera, Isoptera* and *Odonata* (Stys and Blinksi, 1990). The other two ovarian types are characterized by

significant participation of nurse cells or trophic tissue, thought to be derived from germ cells, in the maturation of the oocytes. In telotrophic merositic ovaries the nurse tissue is in the form of a syncytial germarium that forms the distal tip of the ovariole. The developing oocyte remains attached to the germarium by this cytoplasmic nutritive cord (Heming, 2003; Klowden, 2002) through which RNA, ribosomes, proteins and other materials pass into the maturing oocyte. Finally polytrophic meroistic ovaries are found in many holometabolous insects (Stys and Blinksi, 1990). In these ovaries the oocyte is surrounded by a group of sibling nurse cell whose function is similar to the germarium of the telotrophic ovary (Heming, 2003; Klowden, 2002). This group of cells is located at the posterior end of the oocyte and contributes to oocyte maturation, remaining active in synthesizing and passing nutrient material to the oocyte until at last they empty their cell contents into the oocyte before it exits the ovariole to the lateral oviduct.

For each oocyte to successfully produce a hatchling it must contain sufficient stores of lipid and protein in the form of yolk to support embryonic development. In the case of the meroistic ovaries the nurse cells contribute significantly to the stores of the oocyte (Heming, 2003; Klowden, 2002). In the panoistic ovary of grasshoppers the development of yolk reserves depends entirely on synthesis by the oocyte and the follicle epithelium and uptake of reserves from the hemolymph, such as vitellogenin (Vg) and lipophorin (Lp) that are transcribed/translated in the fat body (Fig. 1.2). Vg and other reserves are secreted into the hemolymph and enter the oocyte at the follicle

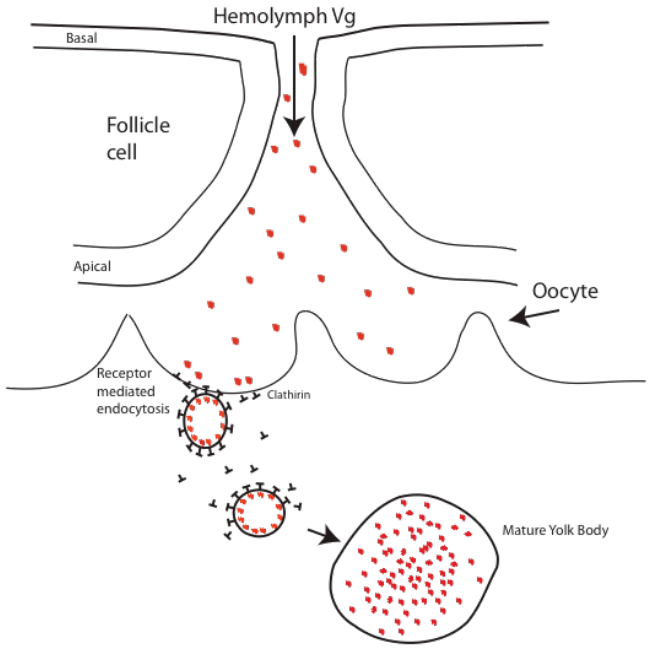


Figure 1.2: Follicle Cell patency and oogenesis
Passage of Vg through the intercellular patent space and its entry to the oocyte by receptor mediated endocytosis. Reproduced from Raikhel, A.S. (1984).

epithelium-oocyte interface (Heming, 2003; Klowden, 2002). In order for this process to proceed, the follicle epithelium undergoes a juvenile hormone (JH)-dependant process referred to as patency. JH initiates this process by the activation of a Na+ / K+ - ATPase that triggers a morphological reorganization of the follicle epithelium to form large intercellular channels through which Vg and other proteins pass to the egg surface (Davey, 1981; Davey and Gordon, 1996; Pszczoloski, 2005). Upon encounter with the microvillae of the oocyte surface, Vg is incorporated into pre-yolk bodies by receptor-mediated endocytosis. The hemolymph-soluble vitellogenin in these pre-yolk bodies is then further processed to its crystalline form, vitellin, for storage as a mature yolk body (Marsh and McMahon, 1999).

M. sanguinipes that have performed as least one long duration flight show greater lifetime fecundity and reduced time to first oviposition relative to grasshoppers that have been identified as migrants willing to make a long duration flight but not allowed to do so (McAnelly & Rankin, 1986b; Rankin & Burchsted, 1992; Min et al., 2004). Min (et al, 2004) observed a transient increase in JH III titers following long duration flight experience and demonstrated that exogenous treatment of grasshoppers with JH III was sufficient to mimic flight experience in stimulating the reduced time to first oviposition. The possible role of JH III in stimulating onset of oogenesis after long flight via its induction of vitellogenesis is easy to envision (Engelmann, 1970). What is not as clear is how a transient peak in JH III might alter life-time fecundity. Experience of flight could have a long-term as well as a short-term effect on oogenesis by triggering endocrine signals that simultaneously accelerate the first round of oogenesis while increasing sensitivity of the ovary so that subsequent rounds of oogenesis would be altered. One possibility is that exposure to JH may alter the sensitivity of the ovary to subsequent JH exposure. One of the goals of this study is to investigate whether a flight-induced peak in circulating JH III might produce this effect; we test this possibility by measuring the effect of JH III treatments on the induction of patency.

Lipophorin, flight and the immune response

The consequences of flight are not likely to be confined to the reproductive timing of oviposition and oogenesis. During flight, lipid mobilization to power the flight muscles is initiated by the release of adipokinetic hormone (AKH) from the corpora cardiaca (Arrese et al., 2001; Min et al., 2003). AKH initiates a signal cascade for the conversion of stored triacylglycerol into diacylglycerol (DAG) and its loading into high density lipophorin (HDLp) during the conversion to low density lipophorin (LDLp) (Reviewed in Arrese and Soulages, 2010). DAG is transported to the flight muscle by the lipophorin complex (Lp) where it serves as fuel for the flight muscles (Kent et al., 1997). Lp conversion between the high and

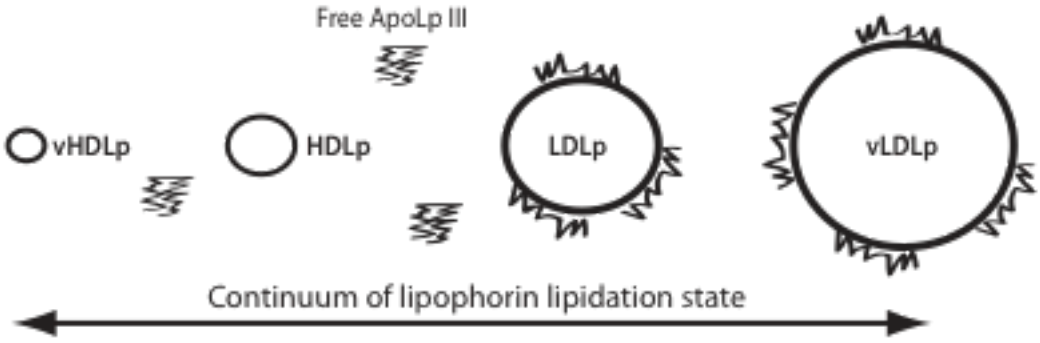


Figure 1.3 Lipohorin particle
Lipophorin exists in the hemolymph of the grasshopper in various sates forming a continuum of sorts between the relatively lipid free vHDLP and the highly lipid loaded vLDLP.

low density states is facilitated by the conformation change and incorporation of the exchangeable apolipohorin III (ApoLp III). ApoLp III incorporation facilitates the loading of hydrophobic diacylglycerol into HDLp to form LDLp (Soulages et al., 1996; Ryan and Vanderhorst, 2000; Vanhoof et al., 2002). Lp is a polymorphic lipid particle that has been observed to contain and transport a wide

variety of lipids and proteins. It is a multifunctional protein-lipid complex involved not only in transporting DAG from the fat body to flight muscle during flight but also in the immune response (Kawoaya and Lau, 1988; Fan et al., 2002; Zieglar and Antwerpen, 2006).

The immune response of insects to the presence of a foreign body is dependent on first recognizing the invader as non-self and thus potentially a threat (Ragan et al, 2009). The cellular envelope of some of the most common insect pathogens such as bacteria and fungi consist of such epitopes as ß-1-3-glucan, peptidoglycan and lipopolysacharide all of which can bind to pattern-recognition proteins present in insect hemolymph (Kurata et al., 2006). The binding of these antigenic molecules leads to initiation of an immune response. Recent discoveries have revealed that the lipid particle of the HDLp-LDLp lipid transport system is involved in immune surveillance and delivery of immune response elements (Rahmen et al., 2006).

Among the elements of the immune response found in association with the Lp lipid particle of the hemolymph is phenoloxidase (PO) an enzyme that catalyzes the oxidative reactions of melanization important in defense against microbial infection (Mullen and Goldsworthy, 2003; Li et al, 2003; Cerenius et al., 2008). PO is present in the hemolymph as an inactive zymogen of PO molecules referred to as pro-phenoloxidase (proPO) (Kanost and Gorman, 2008). ProPO is converted from its inactive state to active PO by a series of proteases well described in *Manduca sexta* (Tagan et al., 2009). The protease cascade

activation of PO is regulated by the presence and titer of a number of small peptides known as serine protease inhibitors or serpins (Tong and Kanost, 2005). These peptides inhibit specific steps in the cascade leading to PO activation.

Coagulation is another element of the immune response in which Lp has been found to participate. Coagulation is a rapid process in which elements of the cell free immune system and cellular elements (hemocytes) act together to close injured cuticle, encapsulate large foreign bodies and heal subcuticular tissues (Karlsson et al., 2004; Schmidt et al., 2009). Coagulation, or the formation of a clot, begins with the attendance and adhesion of procoagulants at the site including Lp (Karlsson et al., 2004; Bidla et al., 2005). Hemocytes and additional coagulants arrive next and form a soft mass of cells and lipid particles. Phenoloxidase is then activated and through its crosslinking enzymatic action, it generates antimicrobial reaction products and aids in the maturation of the clot into a hard surface not unlike mature cuticle (Li et al., 2002). It is interesting to note here that when transformed into an adhesive lipid particle Lp acts as a link between the cell free immune responses (PO), the cellular (hemocytes) and is itself a kind of proto-cell with a structure similar to that of a cellular envelope; consisting of lipid, embedded proteins and carrying elements of the immune response (Schmidt et al. 2009).

A goal of this study is to examine the effect of long-duration flight performance on the circulating titers of major hemolymph proteins including the lipophorins and PO as well as to identify and test the resistance of *M.*

sanguinipes to a novel microbial pathogen following long-duration flight experience.

Flight cost compensations

The phenomenon of flight enhanced reproduction in *M. sanguinipes* presents questions regarding the mechanism by which performance of a long duration flight by females that acts to reduce duration of oogenesis. Locusts have been shown to differ in their nutritional regulation in a phase dimorphic manner (Simpson et al., 2002). Wing dimorphic crickets also show differential resource allocations based on their respective wing state while their rates of ingestion remain the same (Mole and Zera, 1993). One of the goals of this study is to determine whether female grasshoppers compensate for the implied resource costs of flight by increased feeding, improved digestion/food utilization or by increased mating activity.

Mating frequency and duration represent a possible avenue of compensation for female *M. sanguinipes* because during copulation males can transfer as many as 14 nutrient-rich spermatophores (Friedal and Gillot, 1977). Females may obtain additional nutrients via increased frequency of copulation or by increasing the duration of copulation. Mating in *M. sanguinipes* begins by the approach of the male to the female and the performance of a copulatory leap during which he affixes himself to the back of the female (personal observation). The female may either accept or reject the advance of the male during the

subsequent species recognition phase when the male cerci act as a key of sorts to successful copulation (Capinera et al., 2004).

This dissertation sought to expand the work of McAnelly, Burchsted and Min on the reproductive and migratory physiology of *M. sanguinipes*. By examinations of the hemolymph, its components and aspects of ovarian development, in order to expand understanding of the consequences of flight on the hemoylmph proteome, its interface with immune function and how flight experience impacts developing oocytes in the migratory grasshopper *M. sanguinipes*.

Chapter 2

Metabolic and behavioral compensations for flight

Introduction

Migration has often been assumed to involve costs associated with the metabolic resources consumed during flight as well as a delay in time of first reproduction since migratory flight typically occurs in the young adult prior to oviposition (oogenesis-flight syndrome (Rankin and Rankin, 1980)).

In contrast, *M. sanguinipes* that experience a long-duration flight in the laboratory typically show accelerated oviposition and enhanced reproductive success (McAnelly, 1986b; Burchsted, 1991; Min, et al., 2004). One explanation for this counter-intuitive observation might be that *M. sanguinipes* compensate for the resources expended during migratory flight by increasing ingestion or utilization of food. Such a strategy would be an adaptive advantage in a colonizer encountering new habitats and resources (Dingle, 1996). The effect of long duration flight on appetite is an understudied aspect of the life history of migratory insects although wing dimorphism in the cricket *Gryllus reubens* has been examined in the context of nutritional resource allocation (Mole and Zera, 1993). The tradeoffs examined by Mole and Zera were ontogenetic in nature, however, and not demand-mediated as would be the case in *M. sanguinipes.* The capacity of some acridid insects to compensate for dietary deficiencies has been well established by Joern and colleagues (Joern and Behmer, 1998).

Melanoplus differentialis has been shown to compensate for dietary dilution by increasing meal frequency while meal size remains unchanged (Yang and Joern, 1994). Locusts also exhibit a striking phase polymorphic capacity to make resource allocation decisions based upon food quality and quantity (Simpson et al., 1990; Simpson et al., 2002).

Juvenile hormone has been shown to play a central role in the enhancement of reproduction following experience of long duration flight (Min et al., 2004). I therefore used JH treatment as a proxy for performance of flight to investigate whether it might play a role in the compensation for flight. The exogenous application of JH has been shown to raise circulating levels of JH for 24 hours (Min et al, 2004). This rise is similar to that observed following long duration flight.

Male *M. sanguinipes* transfer nutrient rich spermatophores to the female during mating in addition to sperm. (Friedal and Guilloit, 1977; Engelmann, 1970; Cheeseman et al., 1989). Males synthesize protein and lipid rich spermatophores from their respective accessory and white glands (Cheeseman et al., 1988). The content of this copulatory gift varies with the quality of the male and duration of copulation (Belovsky et al., 1996; Hinn and Niedzlek-Feaver, 2001). It is possible that female *M. sanguinipes* may compensate for the resource costs of migratory flight by increasing the frequency and/or duration of copulations, thereby increasing male contribution to oogenesis after flight.

Any or all of these potential compensatory mechanisms could play a role in the life history of a colonizing phytophagous insect and would help to explain the phenomenon of flight-enhanced reproduction.

Methods

Experimental Animals

M. sanguinipes used in these experiments were collected and reared as previously described (Kent, 2001; Min et al., 2004). The animals were first generation offspring of field animals collected on the San Carlos Apache reservation northwest of Globe Arizona.

Tethered-Flight test assay

Grasshoppers were assayed for flight experience and migratory tendency as previously described (McAnelly and Rankin, 1986). Briefly, each grasshopper was attached to a small stick with wax and suspended in front a fan, an electric heater and incandescent lamps in simulation of the conditions necessary to initiate migratory flights in the field (Parker, et al. 1955). Grasshoppers were flight-tested on days 4, 6 and 8 (Day 0 was defined as the date of eclosion). Some grasshoppers were flown only on day 4, while it took others until day 6 or day 8 to fly for more than sixty minutes continuously. Those that flew on day 6 or 8 were discarded from the experiment in order to synchronize the date of flight. Once classified as an LF-1 migrant stopped at one hour of flight of an LF-E migrant allowed to fly to exhaustion a grasshopper was not flight tested again.

Feeding rate determination

Eclosing females were provided with water but denied food for 1 day (day 0). Pellets had a mean wet weight of 0.6801g ± 0.0092g SEM. By weight/volume the pellets contained 4% agar, 3.8% Casein, 2.5% powdered alfalfa, 2.5% sucrose, 1.9% yeast extract, 1.9 % soy bean protein, 1.3% oat bran, 1.3% wheat germ, 1.3% cellulose, 1% Wesson salts, 0.75% corn oil (as antifoam agent), 0.5% ascorbic acid, 0.3% cholesterol, 0.2% vitamin supplement, 0.15% glycine, L-cysteine, choline chloride and double deionized water. Following sterilization methylparaben was added at 0.8% w/v to prevent fungal growth. Single pellets were weighed, placed in small petri dishes nestled between two dental wicks, moistened with 0.1% methyl paraben to prevent drying, fungal growth and increase palatability in the dry heat of the environmental chamber prior to being given to each female. For the determination of feeding rate a standard wet-to-dry weight ratio was determined for each batch of diet pellets (mean 0.2531 ± 0.0023).

Collection of weight data

Each time a new pellet was added, the previous pellet was collected, labeled, dried over night and weighed. When replacing the pellet, the concurrent accumulated excrement or “frass” was collected, dried in the same manner as the pellet and stored for analysis at 5^{o}C. Each grasshopper was also weighed on

an analytical balance each time a food pellet was replaced as well as on the final day of the experiment.

Excrement analysis

Frass samples of known weight were fractionated via a modification of the methods of Handel (1965). Frass was softened in 1ml of a 0.1M KCl solution and sonicated for one minute. One ml of chloroform was added and the solution vortexed for 1 minute. This solution was then centrifuged at 1000 g for 5 minutes. The resulting two phases of liquid were sampled: the chloroform phase was analyzed by vanillin assay for lipid using cholesterol as standard (Burchsted, 1990). The aqueous phase was analyzed for carbohydrate and protein content using a colorimetric anthrone assay with glucose as standard for carbohydrate and the colorimetric Bradford assay (Sigma) with bovine serum albumin as a standard for protein.

Calculations

In order to measure feeding and digestion in *M. sanguinipes* the classic model of Waldbauer (1968) was used to determined the feeding rate, approximate digestibility (%AD) efficiency of conversion of digested material (ECD) and the efficiency of conversion of ingested material (ECI). Feeding rate is reported per milligram of body weight: **FR** = (g dry weight food consumed / days with pellet / mg body weight) Efficiency of conversion of ingested food to body matter**: ECI** = (weight gain / food consumed) x 100. Efficiency of

conversion of digested food to body matter calculated as: **ECD** = (Weight gain / weight of food ingested – weight of feces) x 100%. Approximate digestibility**: AD** = (g of food consumed – g of frass) / (g of food consumed). All calculations performed are adapted from the methods of Mole and Zera (1992), adapted from Waldbauer (1968).

Mating frequency and duration

Females of different flight experience (LF-E and LF-1) were placed in plexiglass observation arenas and observed by Time Lapse Video Recorder (AG-TL950 Panasonic, Minneapolis MN) for seven days following flight experience. Three male grasshoppers of simliar age were placed with each female and the number and duration of mating events was observed. Nocturnal observations were achieved by using illumination with a red spectrum light reported to be outside the visual range of grasshoppers (Kong et al., 1980). TLVR was used for playback and data collection. Data were analyzed by one-way ANOVA

Male spermatophore contribution

Fluoroscein Dextran molecular weight 10,000 (FD) was investigated as a potential tracking dye to assess the contribution of male *M. sanguinipes* spermatophores to oogenesis. 5 μl of a 1 μg / μl solution of FD was injected in to the hemocoel of Day 5 male *M. sanguinipes.* Injections were performed between the second and third abdominal sternite. Males (n = 4) were sacrificed after 12, 24, 48 and 72 hours to determine the location of the FD in their respective reproductive tracts. Another set of FD injected males was mated, and after 24

hours, the female and male were sacrificed and their reproductive tissues examined for the presence and intensity of FD. Following sacrifice and dissection the tissues were fixed in 3.7% para-formaldehyde for 1 hour, rinsed three times in PBS and reserved for imaging. Imaging was performed using a Leica MZ 16 in collaboration with the Microscopy Core Facility of The Institute for Cellular and Molecular Biology of the University of Texas at Austin.

Results

Resource Compensation

The results of these experiments indicate that there is no increase in the mean consumption of food as a compensation for the resource costs of flight (Fig. 2.1). The compositional frass analysis did not show any significant differences between the LF-E, LF-1 or NF groups (protein $f = 0.42$; $df = 30$; $P = 0.663$) (lipid $f = 0.44$; $df = 30$; $P = 0.646$) (Carbohydrate $f = 0.2$; $df = 30$; $P = 0.820$). A significant increase in mean efficiency of conversion of ingested material was observed between the LF-E individuals and the LF-1 control groups($t = 1.94$; $df = 29$; $P = 0.042$) (Table 2.1).

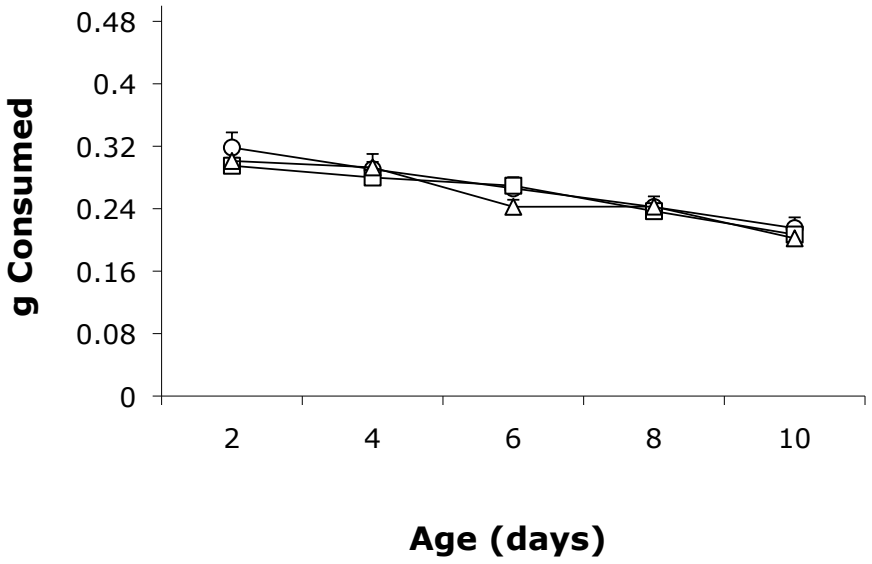


Figure 2.1: Mean feeding rates.
No differences were detected between flight performance categories. Consumption is expressed as grams of food consumed per 2-day interval. Bars indicate standard error of the mean. Square = LF-E, Diamond = LF-1 Circle = NF

Table 2.1. Mean (SEM) nutritional indices of respective flight groups.
LF-E ECI was significantly increased when compared to controls (t = 1.94; df = 29; P = 0.042).

Flight Group	%AD	%ECD	%ECI
LF-E	45.35 (2.75)	22.97 (7.15)	15.26 (0.61)*
LF-1	47.50 (2.79)	57.30 (22.8)	13.63 (0.57)
NF	47.61 (1.64)	30.32 (4.71)	12.57 (1.12)

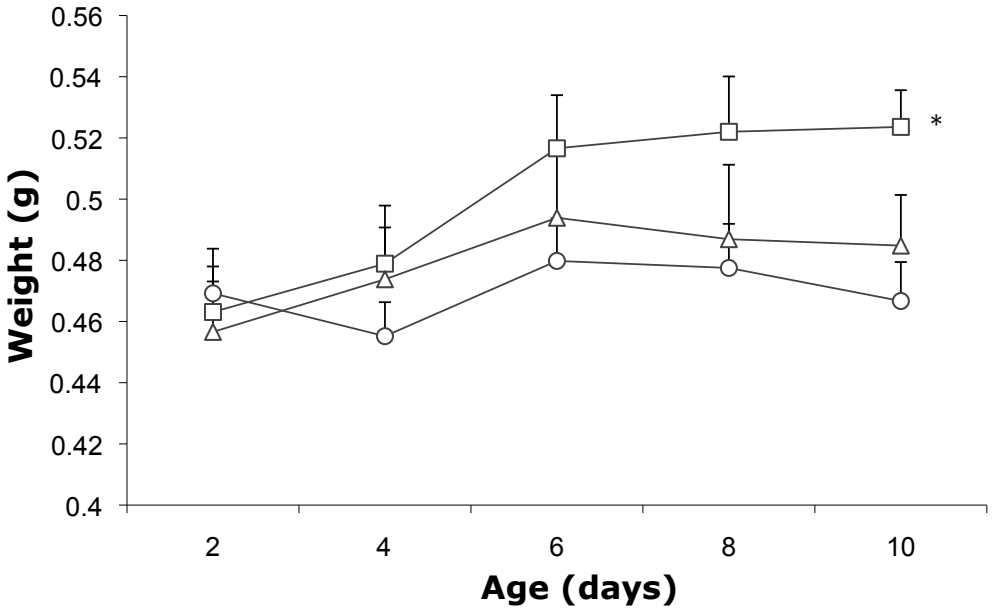


Figure 2.2: Mean body weights
Significant increase in mean body weight change was observed in LF-E (median 0.6112; n = 12) grasshoppers compared to controls (LF-1 median 0.5150; n = 14) by day 10 (Mann-Whitney p = 0.0422). Bars represent standard error of the mean. Square=LF-E, Diamond=LF-1 Circle= NF

Weight gain

Female *M. sanguinipes* gained significantly more weight following flight than individuals that performed a one-hour diagnostic flight or those that refused to fly (Fig 2.2). Digestive indices indicated no difference between the three groups in the amount of frass produced, but conversion indices indicate an increase in the mean ECI of the females that performed a long-duration flight (Table 2.1). This increase in the efficiency of conversion of ingested material is reflected in the increase in weight gain of the females experiencing long-duration flight.

Mating frequency and duration

Over the period of 7 days female *M. sanguinipes* displayed no significant difference in the mean duration or frequency of copulations between the three flight groups (duration F = 0.3701; df = 30; p = 0.694) (frequency F = 1.182; df = 30; p = 0.321). More than 50 % of mating occurred during the light phase of the L:D cycle (Fig 2.3).

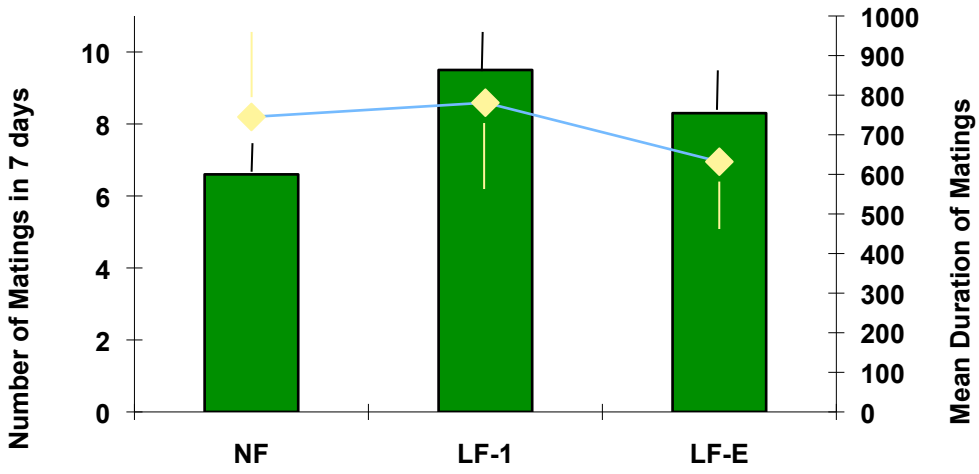


Figure 2.3. Mating frequency and duration.
Mean duration (green) and frequency (yellow) of copulations in *M. sanguinipes.* Bars indicate standard error of the mean. No significant differences were observed in either duration (1 way Anova: F = 0.3701, df = 30, P = 0.694) or frequency (1 way Anova: F = 1.182, df = 30, P = 0.321). Bars represent standard error of the mean.

Male spermatophore contribution

Transport of the tracking dye into the testis through the seminal vesicle to its final accumulation in the ejaculatory duct could be followed after injection of

FD into the male hemocoel (Fig. 2.4 A-C). Males that were allowed to mate following the injection transferred the FD into the female along with the spermatophore. The dilution of the fluorophore combined with a high level of auto fluorescence of the female spermatheca at long exposures proved to be a barrier to imaging the partition of the male contribution.

When FD was used to track male accessory gland products transferred to females during copulation, no difference between flight groups in the amount or destination of contributed material was observed. However, we were able to show that dextran-conjugated fluorescein introduced into the male hemocoel will find its way into the developing eggs of a female with which the male mates (Fig. 2.4). Although we were not able to consistently detect the fluorophore in the spermatheca, its presence in the operculum of the oocytes (Fig. 2.4 D) indicates that this method of paternity tagging may be useful. The data also clearly show a passage of material from the hemolymph into the testis and transfer to the ejaculatory duct via the seminal vesicle (Fig 2.4 A-C). It appears that FD placed in the hemolymph passes into the apical compartment of the sperm tube via the basal half, enters the seminal vesicle and is incorporated into the developing spermatophore. This result is in contrast to that of Szollosi and Marcaillou (1977) who postulated a “blood-testis barrier.” This observation may be a result of the different nature of the tracking dyes used. Szollosi and Marcaillou (1977) found that the basal half of the sperm tube is impermeable to molecules such as horseradish peroxidase placed in the hemolymph.

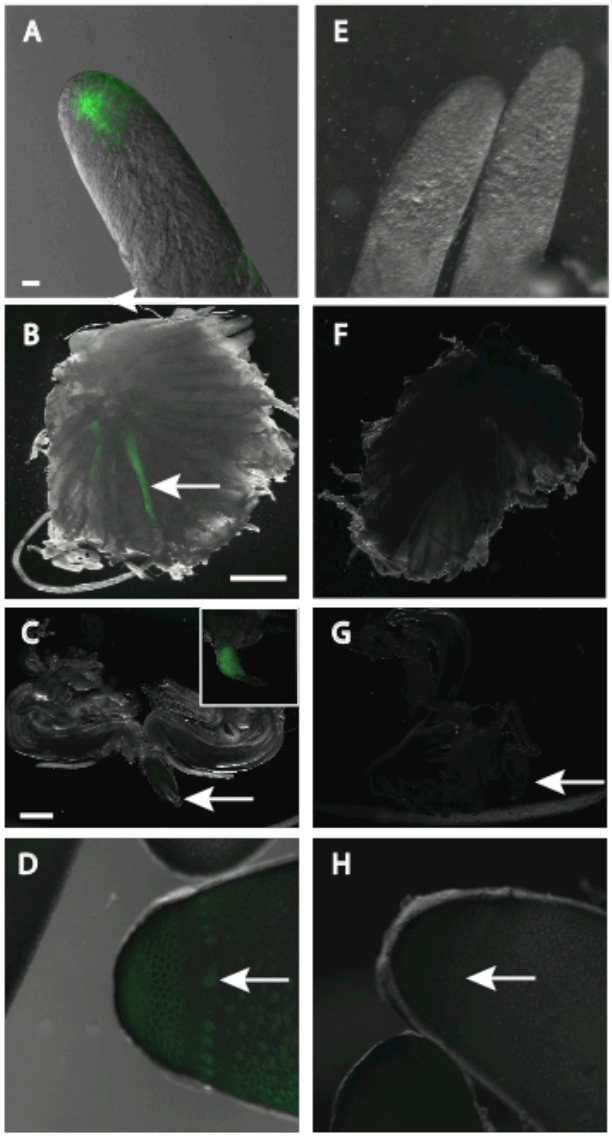


Figure 2.4: Male spermatophore tracking.
Tracking fluorescein dextran through the male reproductive tract of *M. sanguinipes*. FD enters at the apical compartment of the sperm tube (a). Passing through the seminal vesicle (b) it enters the ejaculatory duct (c). FD was detected in the operculum of developing eggs in females with which the FD injected male copulated (d). E – H sham injected controls. A & D scale bars = 100μm B & C scale bars = 1000μm

Discussion

Dietary compensation

The primary aim of this study was to examine whether female *M. sanguinipes* that experience long duration flight and subsequent accelerated oogenesis compensate for resource utilization during flight via increased feeding, improved digestion or food utilization, or increased mating frequency or duration.

Migration and reproduction are life history events that both requiring large reserves of energy (Rankin and Burchsted, 1992). Logically, expending a large proportion of energy stores during flight should reduce reproductive output if simple availability of reserves is a limiting factor in reproduction (Kent et al, 1997). This is not the observed effect of long-duration flight in *M. sanguinipes*, however, so the question arises as to whether such flight experience triggers a change in behavior and/or metaboism such that compensate for the energetic costs of flight. The observation that long-duration flight results in increased weight gain seems again to argue for such an effect. In this study, however, we have shown that over a defined period of time after flight, animals that performed a long-duration flight do not eat or mate significantly more than migrants who experience only one-hour of flight or those who refuse to fly.

All the flight groups were approximately the same average weight at the beginning of the experiment, and other than a slight and not significant increase in food consumption in the preceding interval there seems to be no source for the observed weight gain. There is no increase in mating frequency or mating

duration to provide an alternative source of resources in long-fliers. Thus we are forced to conclude that utilization of resources in food is altered in females that experience long duration flight such that these animals gain weight faster. Recent data from Chen et al. (personal communication of unpublished data) have shown that ovarian weight gain is accelerated in LF-E female grasshoppers. This is consistent with the previous observations of accelerated oviposition in grasshoppers after performance of a long-duration flight. We did not distinguish between somatic and ovarian weight gain in these experiments. Regardless of where the wait gain occurred, however, it seems to be the result of greater efficiency in utilization of food—a tantalizing observation that would bear further investigation.

Chapter 3

Population variation in *M. sanguinipes*

Introduction

This report focuses on physiological and morphological variation within putatively allopatric populations of the migratory grasshopper *Melanoplus sanguinipes.* Phenotypic variation present in populations of migratory species such *as M. sanguinipes* that are separated by great distances and geographical barriers may yield insight into regional adaptation, reproductive isolation, mechanisms of speciation and the evolution of migration.

Local adaptation occurs at the physiological and behavioral level in this species (McAnelly and Rankin, 1986; Fielding and Defoliart, 2007). Fielding and Defoliart (2007) showed that subarctic and temperate populations differed in their growth, development, and nutritional physiology. McAnelly and Rankin (1986) demonstrated variation in the propensity to migrate between populations in Arizona, Colorado, and New Mexico. Knowles and colleagues (2000 and 2001) have used regional variation in genomic markers to study putatively allopatric populations of *melanoplines* to understand post-Pleistocene speciation. A sudden radiation of the short-horned, spur-throated grasshoppers in the so-called rocky mountain sky islands began, following the Pleistocene glaciations 0.2 and 0.3 million years ago (Knowles, 1999). These species continue to evolve today

as climate change and habitat fragmentation further isolate populations (Carstens and Knowles, 2007).

This study presents a comparison of morphometrics, physiological characteristics and DNA sequences for two meta-populations of *M. sanguinipes* from eastern Arizona and northern Colorado, examines whether the two populations are phylogenetically distinct and whether reproductive barriers exist between the two populations.

Methods

Experimental animals

Arizona *M. sanguinipes* used for this study were first generation offspring of ~1000 individuals collected by sweep net ~ 5 miles north of Rocky Gulch on the San Carlos Apache Reservation east of Globe, Arizona. Colorado *M. sanguinipes* were first generation offspring of ~1000 field animals collected in Owl Canyon ~20 miles north of Fort Collins Colorado along US Route 287. Animals were reared as described in Chapter 2.

Climate data

Climate data for mean 2007 monthly rainfall and mean monthly high and low temperature for each site were obtained from the Western Regional Climate Center database at <http://www.wrcc.dri.edu/>.

Morphometrics and phenology

Ten individuals of each sex from each population were weighed on a Mettler Toledo analytical balance. Micrographs from each population were taken on a Wild TYP 355110 stereo microscope with an attached Leica firecam version 1.7.1. Ovaries of Arizona and Colorado animals were excised under physiological saline, and the number of ovarioles per ovary was determined. Diapause determinations were made on pods held at 10^{o}C for two months. The pods were deposited in sand by grouped females of each population or individual mating pairs. They were dissected under physiological saline and the embryonic diapause stage estimated following the protocol of Dingle and Mousseau (1994).

Hybridization

Female and male crosses of the AZ and CO populations were performed by placing one male and one female from each population together in a rearing cage with an oviposition cup containing sand moistened with 0.1% methyl paraben. Egg cups were checked for ovipostions daily and placed in a 10°C environmental chamber to undergo diapause. After 2 months the egg pods were placed in adult environmental chambers (L:D 14/10 and 31^{o}C) and observed for their hatching success. Pods that did not hatch were dissected for analysis as described below.

DNA sequencing

Genomic DNA was isolated from AZ and CO femurs using the Wizard Genomic DNA isolation Kit (Promega, Wisconsin). Amplification of mtDNA site COII was performed following the protocol of Chapco and Litzenburger (2003).

Amplification of Melanopline-specific nuclear markers developed by Carstens and Knowles (2006) was also performed. Of the 7 loci tested only Locus 73 produced an amplicon. Briefly the PCRs were performed using PCR Supermix (Invitrogen, California). PCR conditions for mtDNA cytochrome oxidase were 5 ng gDNA, 10μM forward and reverse primers, initial denature 94°C for 5 minutes, 34 cycles at 94°C for one minute, 52°C for 45 seconds, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. PCR conditions for locus 73 were 5 ng gDNA, 5μM forward and reverse primers, initial denature 94°C for 5 minutes, 34 cycles at 94°C for one minute, 52°C for 45 seconds, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. PCR products were purified using the QIAGEN PCR purification kit (Qiagen, Maryland). Purified PCR products were submitted directly for sequencing at the University of Texas ICMB DNA core facility.

Reproductive timing

Female grasshoppers from each population were tested for flight propensity on day 4 following ecolsion. Individuals that made either a long-duration flight or were identified as migrants and stopped at one hour of flight were mated and monitored for time to first oviposition.

Results

Habitats

The local habitats of AZ and CO differ in mean daily temperature and rainfall. The mean difference in monthly maximum temperature between San

Carlos AZ and Fort Collins CO was 7.5° C (± 0.37 SEM). The Mean difference in monthly minimum temperature was 5.7° C (± 0.37 SEM). Yearly rainfall is similar between the CO site (34.24 cm) and the AZ site (35.00 cm) although Arizona has an earlier rainy season with maximum rainfall in May while Colorado has rainfall maximum in July.

Morphometrics

Male and female *M. sanguinipes* of the AZ and CO populations were compared for their respective body weights (Fig. 3.1). Mean body weights by sex for the two populations were: CO male 0.352 g (± 0.014 SEM) CO female 0.389 g (± 0.013 SEM) AZ male 0.579 (± 0.009 SEM) AZ females 0.866 g (± 0.062 SEM). No significant difference was found between sexes within the CO population (t = -1.992, df = 18, P = 0.062).

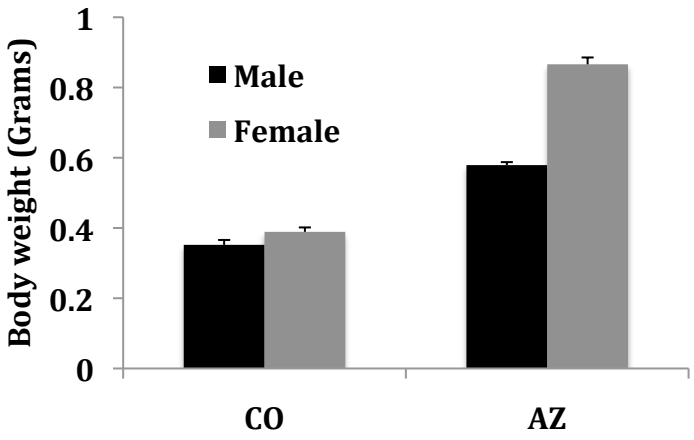


Figure 3.1: Body weight comparisons
Mean body weight comparisons between the sexes of Colorado and Arizona populations of *M. sanguinipes.* Bars represent standard error of the mean.

AZ females did not have equal variance (P < 0.05) with AZ males and were compared non-parametrically by Mann Whitney U. AZ females were

significantly larger than AZ males (U = 0.000, T = 55, P < 0.001). AZ males and females were significantly larger than their CO counterparts (Males; t = -13.926, df = 18, P < 0.001; Females t = -20.518, df = 18, P < 0.001). Some aspects of the external morphology of the two populations were also found to differ between the AZ and CO populations although the male external genitalia, which are considered diagnostic for species identification, were found to be identical in the two species. Wing band pattern and femur band pattern, which are used in pre-copulatory displays (personal observation), were found to be different in the two populations (Fig. 3.2). AZ adults developed a red coloration on the ventral surface of the hind femora and tibia while the CO population retained the hind leg coloration present during nymphal development (Fig. 3.2). The banding pattern of the forewing or tegmen was also found to be different in both size and number of spots (Fig. 3.2).

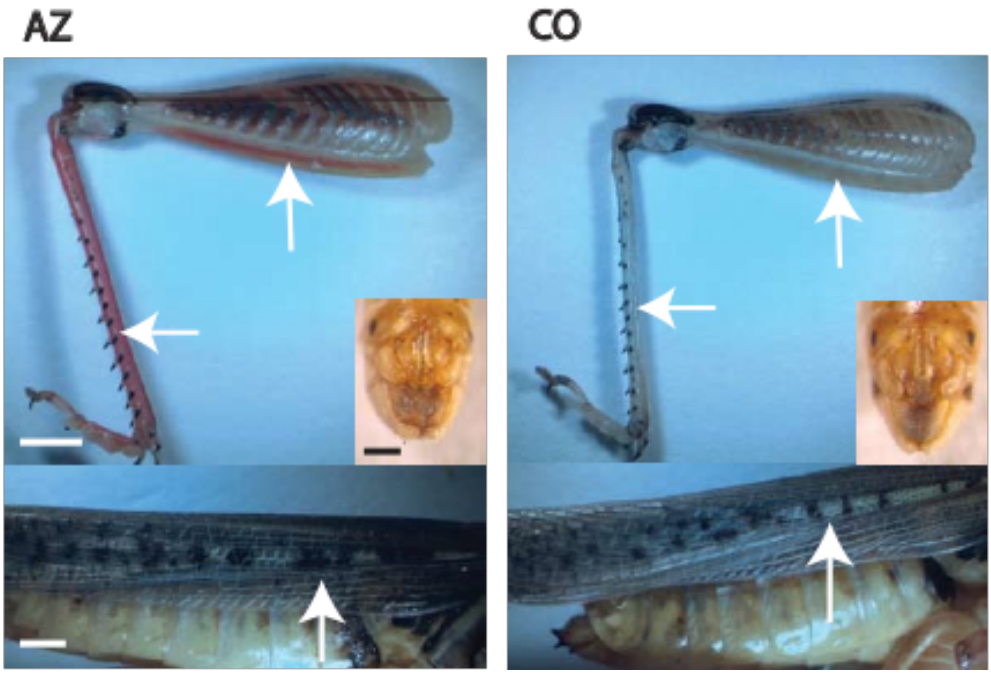


Figure 3.2: External morphological variation.
External morphological variation in Colorado and Arizona populations of *M. sagnuinipes.* Hind leg coloration of the ventral femur surface, tibia and wing spotting pattern differed in the AZ and CO populations. Inset: Male genital characters of the two populations.

M. sanguinipes ovaries from both Arizona and Colorado populations were excised (Chen and Jones, Unpublished data) and the number of ovarioles determined (Fig 3.3 b). The AZ population had significantly more ovarioles per ovary than their Colorado counterparts ($t = -13.574$, $df = 49$, $P < 0.001$). The mean number of ovarioles per ovary in the Arizona population was 37 (± 0.5 SEM) while for the Colorado population mean ovariole number was 24.3 (± 0.9) (Fig. 3.3 b). See figure 3a.

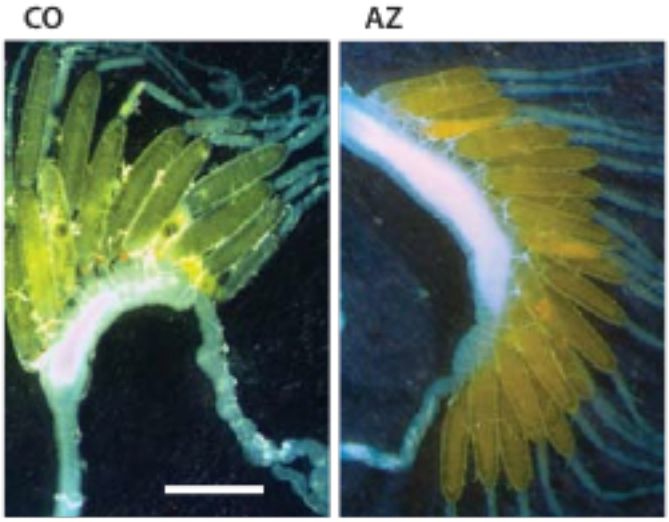


Figure 3.3 a: Representative ovaries.
Micrographs one half of an Arizona and Colorado *M. sanguinipes* Ovary. The lateral oviduct is white with individual ovarioles branching off of it. Scale bar = 3 mm

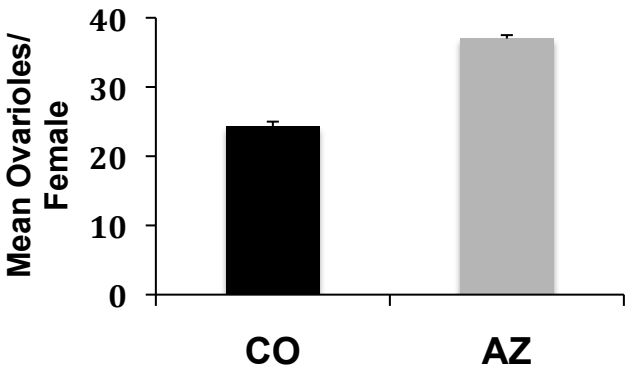


Figure 3.3 b: Mean ovariole number.
Mean ovariole numbers from females of Colorado and Arizona populations of *M. sanguinipes*. Arizona females were significantly larger than their Colorado counterparts. Bars represent standard error of the mean.

Diapause

Egg pods from CO and AZ were held at 15°C for 4 months and individual eggs were dissected under physiological saline to determine diapause state. AZ embryos diapaused at ~stage 23 of development while CO embryos paused development at ~stage 26 (Fig. 3.4).

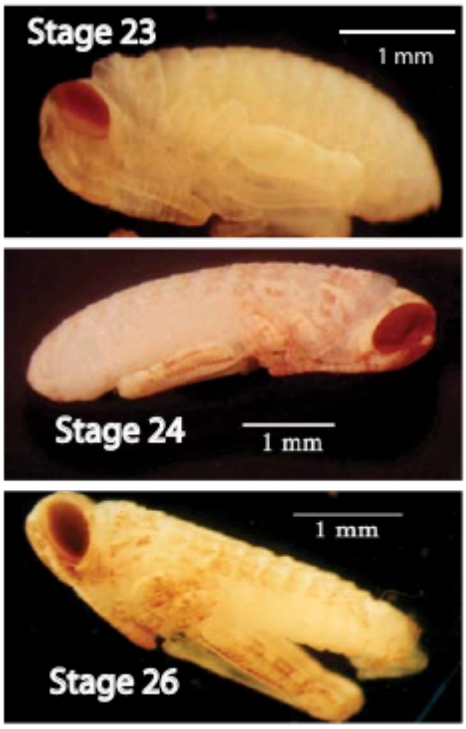


Figure 3.4: Diapause state
Micrographs of diapause states in *M. sanguinipes*. Stage 23 of embryonic development is characterized by partially formed femurs and on overall translucence. CO animals paused development during diapause at this stage. By stage 24 the femurs are more fully formed and the lateral coloration has begun to appear. Hybrids of AZ and CO diapaused at this stage. During stage 26 the femurs of the developing embryo extend to anterior end of the embryo. AZ *M. sanguinipes* entered diapause at this stage (Figure: Saunders unpublished data).

Hybridization

We performed twelve reciprocal mating pairs were set up for AZ and CO. AZ male x CO female yielded 25% oviposition and CO male and AZ female produced 50 % oviposition. In both sets of pairings zero hatching success was observed for the resulting egg pods. The normal oviposition rate for AZ x AZ and CO x CO crosses is between 95 and 100% (unpublished observations). Dissection revealed that hybrid egg pods halted development at ~ stage 24 (Fig. 3.4), intermediate between the stages of diapause observed for CO and AZ. Unlike normal diapause, however, development was not resumed when hybrid eggs were brought to rearing temperature.

DNA sequence comparison

Subunit II of the mitochondrial cytochrome oxidase and one Melanopline-specific variable genomic site of the AZ and CO populations were amplified and sequenced. Sequence homology between the two populations was 99.9 percent (321 bp of COII and 455 bp of Locus 73).

Reproductive timing

Female F1 *M. sanguinipes* from AZ and CO were compared for time to first oviposition following the experience of a long duration flight (Fig 3.5). AZ displayed the previously described reduction in time to first oviposition in LF-E females ($t = 3.043$; $df = 26$; $P = 0.005$) (McAnelly and Rankin, 1986b; Burchsted, 1990; Min, et al. 2001). In contrast, CO *M. sanguinipes* did not show any reduction in latency to first oviposition following experience of long duration flight ($t = 0.067$; $df = 18$; $P = 0.947$) (Fig 3.5).

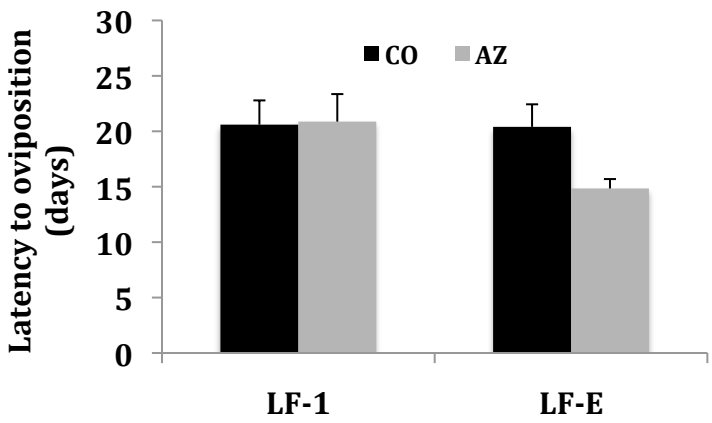


Figure 3.5: Latency to first oviposition
Female *M. sanguinipes* from the Arizona population who performed long duration flight oviposited significantly sooner than females from the Colorado population when compared with females identified as migrants by their completion of a one-hour flight. Bars represent standard error of the mean.

Discussion

We compared variation in physiological, morphological and genetic variation between two populations of the migratory Grasshopper *Melanoplus sanguinipes* from Colorado and Arizona. The two populations were previously reported to vary in migratory tendency (McAnelly and Rankin, 1986a). The stage at which development occurs in this species has been shown to vary considerably over significantly shorter distances than between AZ and CO in relation to altitude (Dingle and Z, 2005).

In these experiments the two populations were significantly different in body weight, number of ovarioles per ovary, embryonic diapause stage and latency to first oviposition. Hybridization experiments showed that both populations readily mated with one another and produced egg pods but hybrid eggs produced in these crosses had zero hatching success. The embryonic diapause state of the hybrid eggs was at stage 24 of development-more developed than the CO population but less than the Arizona population. Yet DNA sequencing and male genital morphology were unequivocal in assigning both populations as *M. sanguinipes*, and McAnelly (1984) previously tested the hybridization of *M. sanguinipes* from Colorado with a population collected in New Mexico and obtained successful F1 and F2 generations from the crosses. The hybridization and hatching success study reported here should perhaps be repeated with a larger control group of like for like pairings in tandem with the inter-population crosses as hatching success has been observed to be reduced

in laboratory reared animals (personal observation). However, the complete failure of the offspring of inter-population crosses to hatch suggests that a post zygotic reproductive barrier may be developing between these populations.

This is the first report of internal morphological and physiological reproductive variation in *M. sanguinipes*. This variation may be an asset in furthering our understanding of the physiological basis of flight-enhanced reproduction. Understanding why the CO population lacks flight-enhanced reproduction while the AZ population consistently displays it will provide a powerful insight into the underlying physiological architecture and possibly the evolution of flight-enhanced reproduction.

Chapter 4

Juvenile hormone and the follicle cell epithelium

Introduction

Small changes in developmental rates (*e.g.*, age at first reproduction) can greatly increase reproductive success (Lewontin, 1965; MacArthur & Wilson, 1967). McAnelly & Rankin (1986b) found that performance of long-duration tethered flight accelerated the onset of reproductive development and oviposition in *M. sanguinipes* (McAnelly & Rankin 1986b). Rankin & Burchsted (1992) subsequently measured the lifetime reproductive success of *M. sanguinipes* maintained as individual pairs. Animals were monitored up to six months post-eclosion for age at first oviposition, lifetime egg pod production, hatching success and longevity. Females experiencing long duration flight (LF-E) were compared with grasshoppers stopped after one hour of flight (LF-1), a length of flight long enough to indentify them as willing to make a migratory flight (McAnelly, 1984). Those experiencing long duration flight began oviposition an average of 10 days earlier than any other group including those flown for one hour.

Min (et al., 2004) tested whether enhanced reproduction after long-duration flight might be dependent on a change in JH production induced by flight experience. He examined daily JH III titers by radioimmunoassay daily in animals that had made a long flight, those that were flown for one hour and those that did not fly; he observed two occurrences of increased titers of JH III in LF-E females shortly after flightrelative to LF-1 controls (Min et al., 2004).

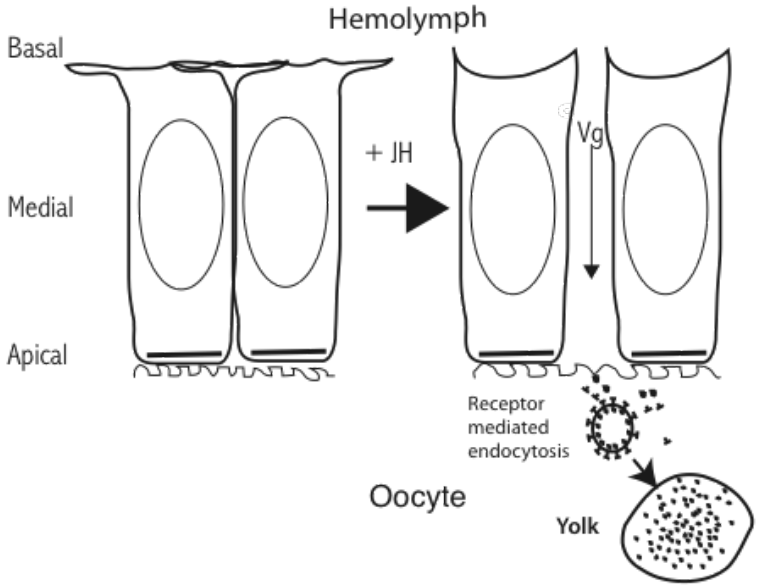


Figure 4.1 Patency and the follicle epithelium
Apicobasal polarity of the follicle cell epithelium and its response to the hormone JH in the process referred to as patency.

One of the most puzzling effects of long duration flight in *M. sanguinipes* is the increase in reproductive success that is maintained over the lifetime of the insect. We know that performance of long-duration flight results in a transient increase in hemolymph JH III titers (Min et al., 2004), and it is easy to understand how these endocrine signals might *accelerate* oviposition in long fliers relative to animals that had not experienced long-duration flight. What is not as obvious is how one long flight could result in a long-term increase in fecundity and reproductive success. One possible explanation is that as a consequence of the cascade of changes that occur after flight to exhaustion, sensitivity of the ovarian follicle cell epithelium to JH III is increased.

As reviewed in chapter one the exposure of ovarioles to JH III initiates a rapid change in morphology of the sheath of follicle cells surrounding the terminal

oocyte (Fig. 4.1) in a process called patency. The process is regulated by JH III through the activation of a Na/K ATPase and Protein Kinase A signaling pathway (Davey and Gordon, 1996; Pszczoloski et al., 2005; Pszczoloski et al., 2008). Patency is an interesting phenomenon in its own right, but it may also provide a clue as to whether or not there are changes in sensitivity to JH III after flight at the target tissue level as well as centrally in response to performance of long flight.

Changes in follicle cell shape have been correlated with changes in the filamentous (F-) actin network present at the midline of the follicle epithelial envelope (Robinson and Cooley, 1997; Fausto et al. 1998; Tanaka and Hartfelder, 2004). Medial intercellular spaces visualized by staining F- actin are a convenient and reliable indicator for assessing patency (Fausto et al. 1998). Here we examine the effect of JH III on ovarian patency and the effect of previous exposure to JH III (as would occur after flight to exhaustion) on ovarian sensitivity to JH III as measured by the patency response.

Methods

Experimental Animals

M. sanguinipes used in these experiments were collected and reared as previously described (Kent and Rankin, 2001; Min et al., 2004). The animals were first generation offspring of ~1000 field animals collected in Owl Canyon ~20 miles north of Fort Collins Colorado along route 287. During these

experiments access to our preferred population from Arizona was limited due to drought and disease.

Statistics

All statistics were performed in Sigmaplot version 11.0.0.77 (Systat Software, Germany). P values are reported directly. Parametric methods (t-Test) were used where the respective assumptions of normality and equal variance were met and non-parametric (Mann Whitney U) if not.

Tissue culture and whole-mount oocyte histology

In order to assess the *in vitro* patency state of grasshoppers following long duration flight a technique for examining the appearance of intracellular spaces between follicle cells was developed. A confocal microscopy method was preferred over the traditional light microscopy methods because I wished to examine the very earliest stages of patency. Tissues were excised under physiological saline. The size of each ovariole within a lobe of an ovary varied considerably. Oocytes that were between two and four mm^2 in area were used for *in vitro* incubations. During *in vitro* incubation oocytes were placed in TC-199 insect tissue culture media containing solvent (N = 7), 10^{-7} M JH III (N = 7) and 10^{-5} M (N = 6) and incubated at 32^{o}C for 2 hours. The oocytes were then fixed in MEMFA (4% fomaldehyde, 0.1M MOPS, 2mM EGTA, 1mM $MgSO_4$ pH 7.4) for 0.5 hours (Lee et al., 2008). Between this and all subsequent steps, tissues were rinsed three times in PBS. Tissues were then permeated in PBS containing 0.01% Triton X-100 and 1% BSA (PBSTB). All whole mount histochemistry

incubations were two hours at room temperature or overnight at 4^{o}C unless otherwise indicated. For F-actin staining, ovaries were incubated in 0.1% Alexa-488 phalloidin (Molecular Probes) in PBSTB. Follicle cell nuclei were stained with 100nm DAPI in PBS for 30 minutes at room temperature. VG uptake by follicle cells was visualized by labeling of vitellogenin in the intercellular spaces of the follicle cell epithelium using a polyclonal primary antibody raised in rabbit against *M. sanguinipes* vitellin (Fig 4.2). Tissues for whole mount immuno-histochemistry were blocked with PBST containing 2% NGS and 2% BSA. Oocytes were then incubated with 1:500 polyclonal rabbit anti-vitellin 1^{o}Ab in PBSTB. Tissues were incubated with rhodamine-goat anti-rabbit secondary antibody at 1:500 dilution in PBSTB (Molecular Probes). Endocytosis at the apical follicle cell surface was visualized by incubation with 4 mM fixable analog of the FM 1-43 membrane probe Fm1-43fx (Molecular Probes) used by Whalley et al (1995) and Brooks and Wessel (2003) to examine membrane trafficking and vesicle transport in oocytes.

Exogenous JH III treatment

To test the effect of exogenous JH III treatment previously shown to be sufficient for enhanced reproduction, 50 µg of JH III dissolved in methanol was applied to the third abdominal sternite of female *M. sanguinipes* (N = 24) on day 7 following emergence. Methanol solvent was applied to the third abdominal sternite of control *M. sanguinipes* females (N=17). These individuals were then dissected in the window of day 17 – 24 and those whose ovaries fell within the 2

– 4 mm^2 range were assayed for their respective *in vitro* follicle cell response to 10 $^{-7}$M JH III incubation or solvent control.

Confocal microscopy

Confocal microscopy was performed on a Leica system model SP2 AOBS at the University of Texas ICMB Microscopy Facility with the exception of Figure 2, which was collected on a Zeiss system LSM 510. Area calculations were performed via the image analysis tool of Adobe Photoshop CS4 extended utilizing the pixel / μm^2 calibration generated by the software of each image collected from each microscope respectively. Basal endocytosis was examined using the fixable analogue of the membrane dye FM 1-43fx with a 488nm excitation and an emission window of 560-650nm. For the analysis of Alexa-488 phalloidin stained F-actin the 488nm laser was used for excitation and the emission window was 508–552nm. Primary antibody bound to Vitellogenin was labeled with goat anti-rabbit conjugated Alexa-534 with a 543nm laser used for excitation and an emission window of 565–599nm. Nuclei were labeled with DAPI (Molecular Probes) with a 405nm laser and the emission window was 433–482nm. Controls using the rabbit pre-immune serum in place of the primary antibody indicated no nonspecific binding. All midline confocal slices were z-plane oriented to the middle of the nuclei of the follicle cells. All micrographs were collected with a 63x oil objective and 3x digital zoom.

Antibody preparation

We raised polyclonal antibodies in rabbit against the 100kD yolk protein vitellin (Fig. 4.2), which has previously been shown to be immunologically indistinct from its hemolymph vitellogenin precursor (Handley et al., 1998). The largest band from homogenized egg was excised from SDS-PAGE gel, homogenized and injected. Antiserum was washed in male blood (5µl cell free hemolymph in 1ml of serum) overnight at 4^{o}C then centrifuged for 10 mins at 10,000 RCF. Western blotting of a 4-12% SDS-PAGE gel of adult female *M. sanguinipes* hemolymph confirmed binding of to a single ~100 KD band present in cell free hemolymph.

Image analysis

Photomicrographs were analyzed for using the CS4 extended (Adobe, San Jose CA). Patency index was determined manually by measuring the total F-actin bordered intercellular area on each sample micrograph using the loop tool of the CS-4 software. Pixel calibrations were provided by the instrument . FM 1-43 intensity was measured by the mean grey value for each micrograph.

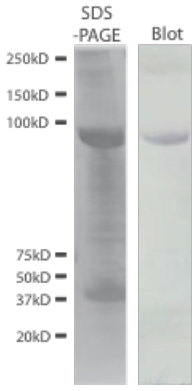


Figure 4.2 Western blot of Vg 1^{o} Ab
Western Blot of primary antibody raised against the yolk protein vitellogenin. Vitellogenin is present in the hemoymph as a ~95kd protein during its transit to the oocyte where it is sequestered as the mature yolk protein vitellin.

Results

Patency in the terminal oocytes

The location of F-actin and Vg in the intercellular spaces of the follicle cell epithelium of vitellogenic *M. sanguinipes* oocytes was determined (Fig. 4.3) using immunohistochemistry. Vg was broadly present in the basal and medial intercellular spaces (Fig. 4.3 a-b). At the apical surface Vg was localized in endocytic vesicles approximately 0.5 μm in diameter (Fig. 4.3 c). Using the weakly lipophilic membrane dye FM 1-43fx, we measured the endocytotic response of the basal surface of *M. sanguinipes* follicle epithelium to JH incubation and compared it with that of methanol-incubated controls. Oocytes incubated with JH III showed a significant increase in the intensity of FM 1-43 labeling on the apical surface of the oocyte (n = 6; mean grey value 25.849 ±3.124) when compared to solvent-treated controls (n = 6; mean grey value 15.18 ± 2.873) ($t = 2.514$; $df = 10$; $P = 0.031$) (Fig. 4.4). This putative endocytotic activity associated with JH III incubation was similar in morphology to that observed at the basal follicle cell surface in vitellogenic oocytes (Fig. 4.2 c).

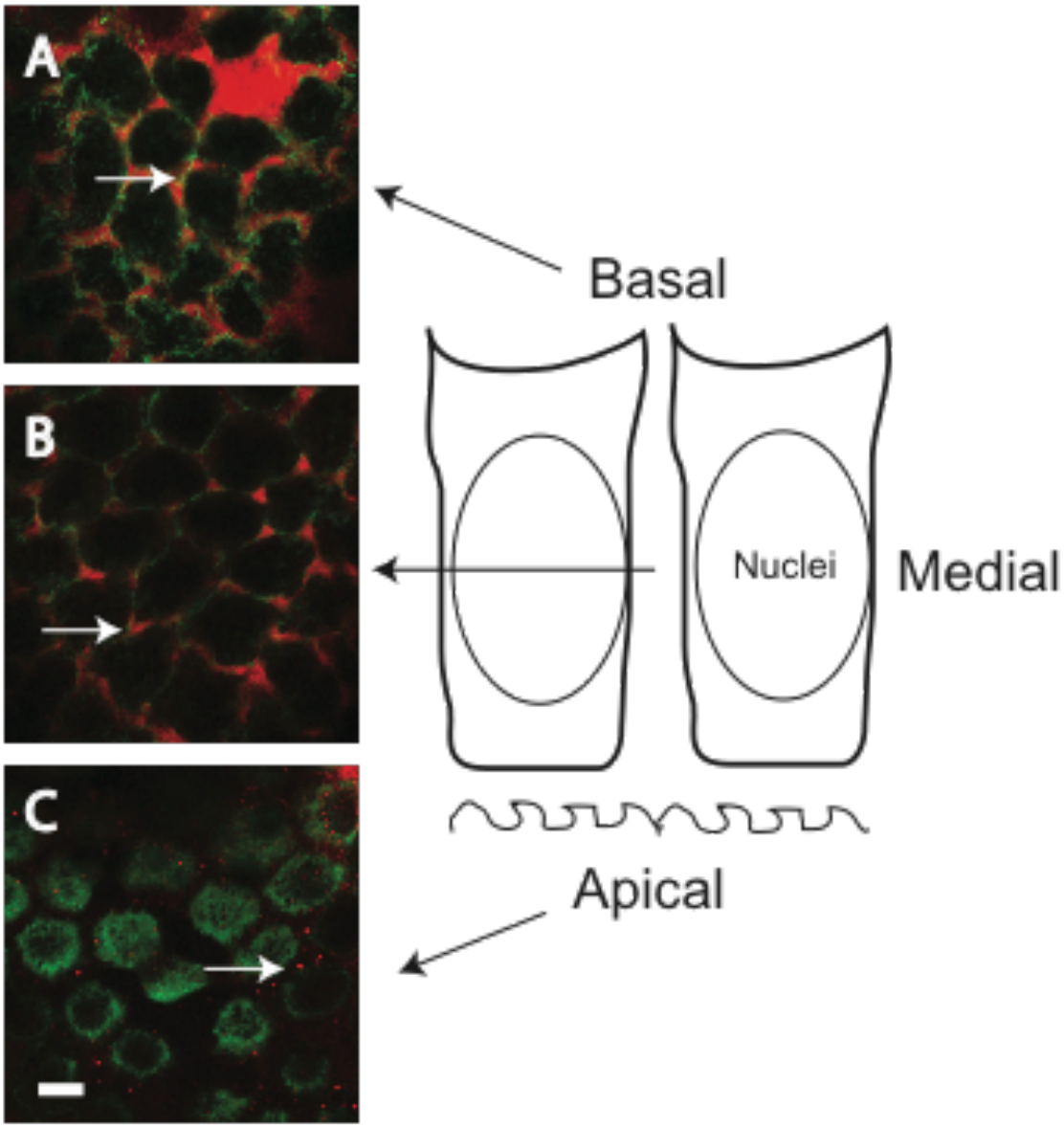


Figure 4.3: F-actin, Vg and patency in the follicle epithelium.
Descending apicobasal polarity of FCE with phalloidin labeled F-actin (green) and antibody labeled vitellogenin (red). Vg passes between the intercellular spaces (A – C) to enter the oocyte via endocytosis (C). Scale bar 10 μm

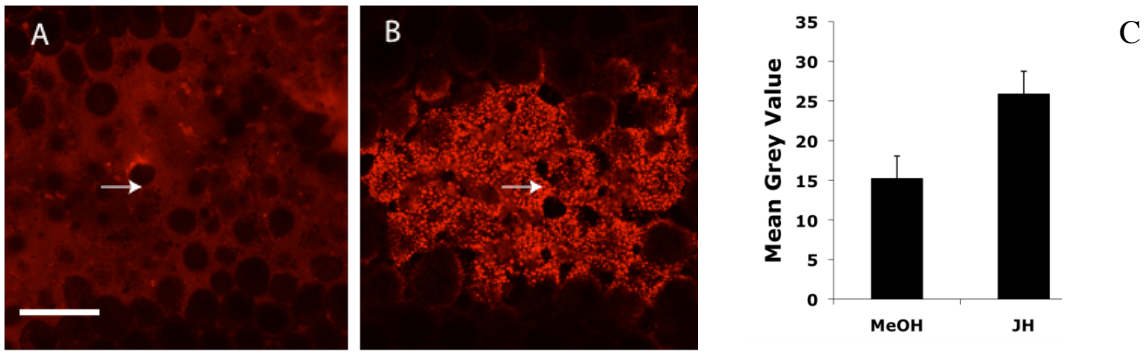


Figure 4.4: Apical induction of endocytosis
Apical endocytosis associated with JH incubation in terminal oocytes of *M. sanguinipes*. Endocytosis tracking dye FM 1-43 red. (A) MeOH incubation (B) 10^{-5} M JH III incubation. Mean grey values for the two treatments (n = 6 each treatment) mean grey value Scale bar 50 μm. Bars represent standard error of the mean.

The follicle cell epithelium of *M. sanguinipes* oocytes also showed an increase in patency in response to *in vitro* JH III incubation (Fig. 4.5). Intercellular spaces showed an increase in size compared to lower JH III treatments or solvent-treated controls.10^{-5} M JH III incubation. Response to 10^{-7} JH III (mean μm^2 129.06 ± 18.21) was not significantly different from solvent-treated controls (mean μm^2 83.73 ± 11.92) with respect to the mean area of intercellular spaces (t-test t = -2.135; df = 13; P = 0.052). The mean intercellular area was significantly increased when JH III treatment was increased to 10^{-5} M JH III (mean μm^2 284.170 ± 54.87) (10^{-7} M JH III mean μm^2 129.06 ± 18.21) (t-test t = -2.864; df = 12; P = 0.015).). In other words, patency was apparent after treatment with 10^{-5} M JHIII but not after treatment with 10^{-7}M.

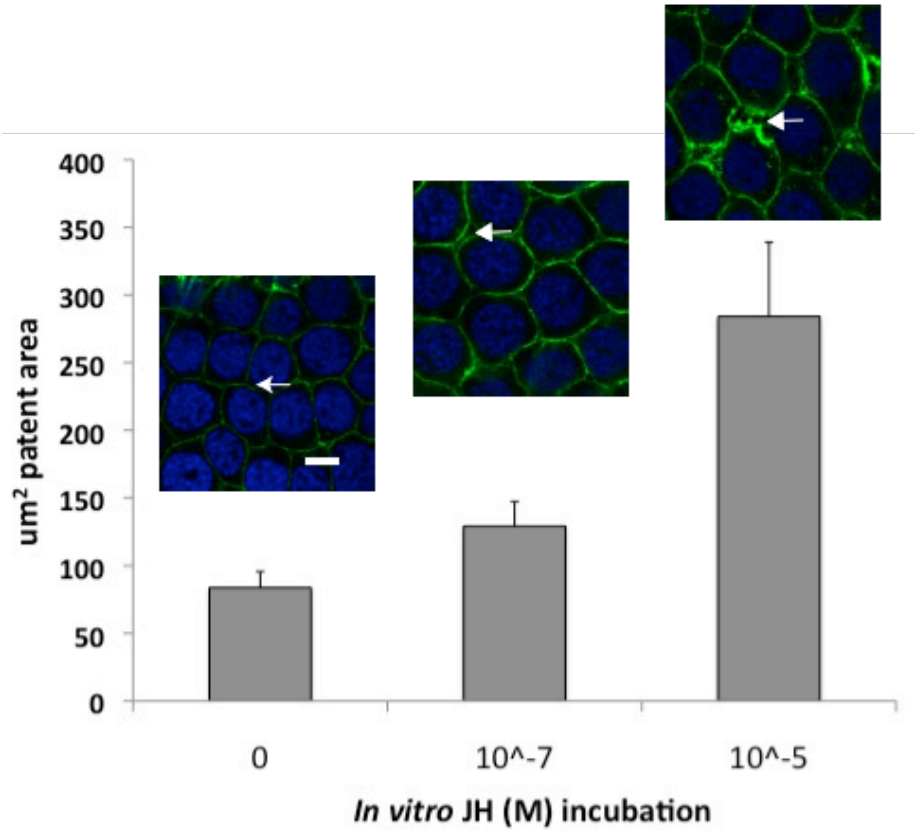


Figure: 4.5 Medial induction of patency by JH III
Medial induction of patency in *M. sanguinipes* follicle cell epithelium. 0 JH III (mean μm^2 83.73 ± 11.92) incubation versus 10^{-7} JH III (mean μm^2 129.06 ± 18.21) was not statistically different in mean patent area (t-test t = -2.135; df = 13; P = 0.052). 10^{-5} M JH III (mean μm^2 284.170 ± 54.87) incubation versus 10^{-7} M JH III (mean μm^2 129.06 ± 18.21) showed a significant increase in mean patent area (t-test t = -2.864; df = 12; P = 0.015). Scale bar 10 μm.

Exogenous JH III treatment and in vitro patency

Exogenous "priming" treatment of females with JH III ~10 days prior to dissection and oocyte incubation increased the patency response of the terminal oocyte to *in vitro* JH III in *M. sanguinipes* (Fig. 4.5). Control ovarioles incubated with solvent showed no significant difference in patent area (Mann Whitney Rank Sum Test T = 54; U = 23; P = 0.902) between JH III-treated (mean μm^2 97.44 ±

20.37) and solvent-treated controls (mean μm^2 109.55 ± 49.58). Oocytes from females that had been treated exogenously with 50 µg of JH III on day 7 displayed significantly higher mean patent area (mean μm^2 274.34 ± 55.57) than those treated with methanol solvent controls (mean μm^2 112.22 ± 22.21) when incubated *in vitro* with 10^{-7} M JH III (Mann Whitney Rank Sum Test T = 71; U = 6; P = 0.017).

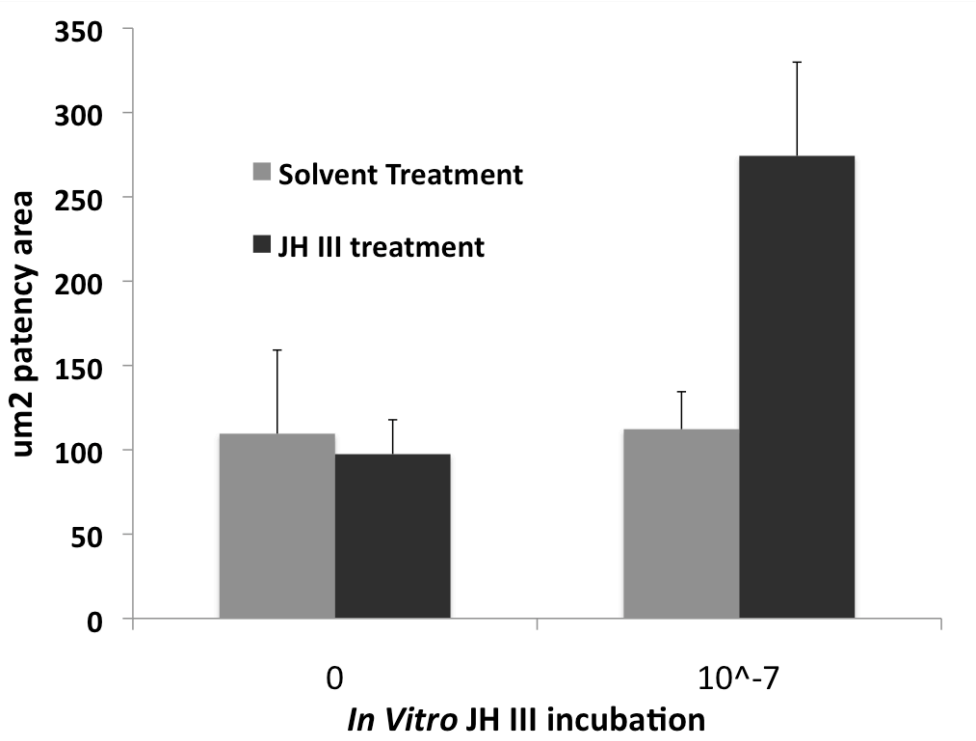


Figure 4.6: Exogenous JH treatment and in vitro induced patency
Oocytes from females who had been treated with 50 µg JH III on day 7 were observed to display significantly higher mean patent area (mean μm^2 274.34 ± 55.57) than those treated with methanol solvent controls (mean μm^2 112.22 ± 22.21) when incubated *in vitro* with 10^{-7} M JH III (Mann Whitney Rank Sum Test T = 71; U = 6; P = 0.017). After incubation with solvent there was no significant difference in patent area (Mann Whitney Rank Sum Test T = 54; U = 23; P = 0.902) between JH III exogenously treated (mean μm^2 97.44 ± 20.37) the MeOH solvent exogenously treated controls (mean μm^2 109.55 ± 49.58).

Discussion

The action of JH on the insect ovary is undoubtedly pleiotrophic, with effects ranging from induction of patency in the somatic follicle cells to the maturation of the oocyte (Davey, 1981). This work was an effort to understand physiological mechanisms underlying the previous work of Min (et al., 2004) that established a central role for JH III in flight-enhanced reproduction. Min's work showing a peak of JH following flight provided a clear physiological connection between performance of long-duration flight and acceleration of oogenesis in the pre-reproductive female. What is still to be understood is how a single long-duration flight followed by a transient increase in juvenile hormone might facilitate a lifetime increase in fecundity. One possibility is that the initial exposure of the ovary to a peak in JH III following long-duration flight causes an increase in sensitivity of the ovary to subsequent JH exposure, possibly via an increase in JH receptors in the ovary and/or other target tissues.

Juvenile hormone is associated with nearly every developmental and life history event in insects (Riddiford, 2008). Finding a receptor(s) for juvenile hormone has proven to be one of the more challenging efforts in insect biology although the action of JH at the level of the gene has begun to be described conclusively (Konopova and Jindra, 2007; Riddiford, 2008).. In this study we have used the indicator of patency to demonstrate a response to JH rather than trying to identify and quantify or characterize JH receptors in the ovary.

We found that the follicle epithelium of oocytes of *M. sanguinipes* responds to *in vitro* incubation with JH by the formation of F-actin-bordered intercellular spaces through which vitellogenin passes before being incorporated into the developing oocyte. In addition, the follicle-oocyte interface was responded in vitro to the presence of JH III by the appearance of endocytotic vesicles characteristic of vitellogenin uptake and packaging during oogenesis.

We showed that when adult female *M. sanguinipes* were pre-treated with JH III on about the day they would have experienced a JH peak if they had made a long-duration flight, the ovarian follicle cell epithelium showed an increase in sensitivity to *in vitro* JH III as measured by the induction of patency. In other words, we used JH III treatment as a proxy for the experience of long duration flight both for the convenience of synchronized study subjects and because our supply of F1 migrant *M. sanguinipes* was interrupted during this study. Indeed pre-exposure of females to JH III via exogenous treatment on about day 7 resulted in a significant increase in sensitivity of the ovary to JH. To our knowledge this study is the first report of JH III acting as a prime to the pump of follicle cell patency. The next step in this analysis will be to examine how the experience of flight impacts the sensitivity of the follicle epithelium to in vitro JH III induced patency. As a life history adaptation the use of JH III in this manner would be a powerful advantage to a colonizing insect seeking to accelerate oviposition following performance of a long duration migratory flight. How this priming occurs at the molecular level is still to be discovered. Whether this action

of JH III is at the level of the genome of the follicle epithelium or via a membrane effect involving the established Na^+ K^+ - ATPase signal will be the subject of future research. If the initial peak of JH after long-duration flight permanently sensitizes the ovary as well as stimulating more rapid initial development, this could be the key to understanding the effect of flight on life-time fecundity in this species—a powerful life history adaptation in a colonizing insect.

We observed the apicobasal polarity of *M. sanguinipes* follicle epithelium to reflect that observed in more advanced meroistic oocytes. The effect of *in vitro* JH III incubation on the follicle epithelium varied along this apicobasal plane and was not confined to the induction of the intercellular spaces of patency that were the focus of this study. In addition to the induction of patency the basal surface of the follicle epithelium of *M. sanguinipies* responded to incubation with JH III by a change in pattern of F-actin cytoskeleton morphology. The basal follicle epithelium surface before incubation was observed to have long F-actin projections connecting cells to those adjacent. These F-actin projections were shortened upon exposure to JH III. It is not clear what role if any these basal F-actin projections might play in either yolk protein sequestration or oocyte maturation. They may have some role in the follicle epithelium involvement in embryo morphogenesis. Lacking the more derived characters of the trophocytic meroistic ovary the follicle epithelium of *M. sanguinipes* must develop without the aid of nurse cells or a nutritive cord. The role of the follicle epithelium in the development of the oocyte would necessarily be larger than that of meroistic

ovaries. No clear conclusions about the role of JH in the basal patterning of the follicle epithelium can be drawn from the data presented here, however it is clear that JH III has a profound impact on apicobasal polarity and that this type of analysis may prove fruitful in the discovery of new information regarding JH action during oogenesis. The next step in this line of research is to examine the effect of JH III on the sensitivity of the follicle epithelium of *M. sanguinipes* over subsequent oviposition cycles and the role of flight experience to determine whether the observed change is maintained during each bout of oogenesis and whether there is a change in some other regulatory network that reduces the threshold of JH III necessary for patency to proceed.

M. sanguinipes display an increase in the sensitivity of the follicle epithelium to JH induction of patency following exposure to JH early in adult life. This change in our understanding of the mechanism of flight enhanced reproduction in *M. sanguinipes* will help further our understanding of it's complex life history.

Chapter 5

Flight performance and hemolymph peptidomics

Introduction

The physiological basis of flight-enhanced reproduction is complex. Min (et al, 2004) demonstrated that changes in the endocrine system after flight are correlated with the phenomenon of flight-enhanced reproduction. As noted previously, performance of a migratory flight leads to a significant transient increase in circulating juvenile hormone III (JH III) titer. Exogenous administration of JH III also accelerates reproduction, mimicking the performance of a migratory flight. It is possible that neuropeptides from brain neurosecretory cells (BNSC) or the corpus cardiacum (CC) may also be released as a consequence of flight and are involved in the observed stimulation of reproduction either via an effect on the CA and JH III release or by independent effect. There is some evidence from the early literature on locusts to suggest that such changes may occur during performance of flight on roundabouts (Goosey and Candy, 1980). In other orthopteran species JH III synthesis and release is regulated by brain neuropeptides released from the corpora cardiaca into the hemolymph (Stay, 2000). A comparative study on the hemolymph peptideomics of grasshoppers that performed flight to exhaustion versus those experiencing a one-hour of diagnostic flight was performed.

As mentioned above, early work with locusts showed that performance of long-duration flight on a roundabout resulted in the depletion of stainable material from the neurosecretory cells of the brain and corpora cardiaca (Highnman and Haskell, 1964). This suggested that performance of flight might trigger the release of endocrine signals from the brain that could potentially regulate a cascade of physiological events following experience of flight. Understanding the changes that take place in the migratory grasshopper's hemolymph during and after flight may be central to understanding the impact of flight experience on the initiation of reproduction. The transmission of neuroendocrine signals via the hemolymph may be the first step of the signal pathway responsible for the acceleration of oogenesis after flight to exhaustion (Engelmann, 1970; Min et al., 2004).

The experience of long duration flight itself results in changes in the hemolymph (Kent et al, 1997) because of mobilization of fuels for flight. The largest initial effect of flight is the depletion of carbohydrate stores by the flight muscle and the subsequent mobilization of fat body lipid via the high density lipophorin (HDLp) low density lipophorin (LDLp) shuttle (Ryan and Van der Horst, 2000). The activation of lipid mobilization during flight is dependant on the a release of adipokinetic hormone from the CC that initiates the loading of diacylglycerol into the LDLp lipid particle. The lipid particle is also known to be involved in the insect's response to foreign material in the hemolymph (Weers and Ryan, 2006).

The hemolymph peptidome has been examined in detail in numerous insects including the phase polymorphic African plague locusts *Schistocerca gregaria* and *Locusta migratoria* (Reviewed in Schoofs et al., 1997). In locusts serine protease inhibitor peptides in the hemolymph and neuropeptides from the corpora cardiaca have been found to vary in correlation with phase (Clynen et al., 2002). Serine protease inhibiting peptides (SPIs) have also been identified in the ovaries of the locust *Scisotocerca gregaria* (Hamdaoui et al., 1998). Serine protease inhibitors are known to inhibit the activation of the phenoloxidase immune response (Tong and Kanost, 2005). Together these findings suggest a widespread role of small regulatory peptides in life history variation and have led us to investigate the role of SPIs in flight-enhanced reproduction in *M. sanguinipes* following long duration flight.

Methods

Experimental animals

All animals used in this study were F1 adult, lab-reared *M. sanguinipes* derived from animals collected yearly on the San Carlos Apache reservation in eastern Arizona. Tethered flight was used to simulate long duration migratory flight and to identify migrants by one-hour diagnostic flight (LF-1) (McAnelly and Rankin, 1986). The one-hour rule has been used to identify an individual as a migrant but to not allow that migrant to perform a long duration flight. Long fliers (LF-E) were allowed to fly on tether to voluntary cessation.

Hemolymph sample preparation

We collected hemolymph by puncturing the dorsal neck membrane between the head and pronotum. Samples were collected on day 6 from females immediately following performance of flight. Hemolymph that bled freely from the puncture was collected in a graduated glass micropipette in the amount of 15 μl for each individual. Hemolymph extraction for HPLC analysis followed the method of Clynen (*et al*., 2002). Briefly, an aliquot of 15 ml hemolymph was combined with methanol/water/acetic acid. After sonication and centrifugation, the pellet was discarded and 100 ml TFA in water was added to the supernatant. The methanol was evaporated and the remaining aqueous residue was re-extracted with ethyl acetate and *n*-hexane to remove lipids. The aqueous phase was filtered through a Millipore Ultrafree-MC 0.1 mm pore centrifugal filter prior to the HPLC analysis.

RP-HPLC of hemolymph

Hemolymph samples were dissolved in 50μl 12% acetic acid prior to injection. Aliquots of hemolymph extracts were injected directly following filtration. The following conditions were used for HPLC on an analytical HPLC system (System Gold, Beckman Instruments, Inc., Fullerton, CA). A 1 x 250 mm C18 column (Separation Technologies Inc.) was used. Solvent A: 0.1 % TFA in water; solvent B: 0.09 % TFA in acetonitrile/water (80/20, v/v). The column was equilibrated with 5% Buffer B and the gradient was developed after sample injections as follows: 5% buffer B for 10 minutes followed by a gradient to 65% B

in 80 minutes, an equilibration step of 65% B for 10 minutes and a gradient from 65% to 5% B in 2 minutes. Absorbance was monitored at 214. Fractions were automatically collected on-line by selecting the drop mode (8 drops/tube) using a Foxy Jr. fraction collector (Isco, Lincoln, NE).

HPLC tandem ESI-MS

Experiments were performed on a liquid chromatography system modified to allow for stable flow rates between 1-200 ml/min (System Gold, Beckman Instruments) linked to an Esquire ion trap mass spectrometer (Bruker Instruments). Electrospray samples were introduced into the mass analyzer at a rate of 2.0 ml/min for online infusions and 1.0 ml/min when using direct infusions with an adjustable syringe pump (Hamilton Instruments). In the standard operating mode, the positive ions, generated by charged droplet evaporation, entered the analyzer through the orthogonal spraying interface of the ion trap.

Sample preparation for peptide sequencing

Reduction / Pyridylethylation. The dried sample was dissolved in 40 µl of 0.1M ammonium bicarbonate buffer, pH 8.5. 60µl of freshly prepared DTT in Urea (2.6 µmol DTT in 10M urea) was added to the sample. The sample was purged with argon, mixed and incubated at 50ºC for two hours. Four µl of 4-vinylpyridine in ethanol (1/6 v/v, freshly prepared) was added and the sample was then purged with argon, mixed and incubated in the dark for 20 minutes. Desalting of the sample was performed using narrowbore RP-HPLC as described above in preparation for sequencing.

N-Terminal Sequencing

Fractions were blotted to a PVDF membrane. The membrane was loaded into the reaction chamber of an Applied Biosystems Procise 492clc protein sequencer for N-terminal sequencing via Edman-degradation following manufacturer's directions.

Results

RP-HPLC analysis

We performed an RP-HPLC analysis of hemolymph extracts from individual female *M. sanguinipes* that performed a long-duration flight (LF-E) and migrants who performed one-hour diagnostic flight (LF-1). The hemolymph extracts of grasshoppers show differences in the chromatographic elution patterns at 214_{nm} UV absorption (Figure 1). Peaks 1 and 2 in Fig. 5.1 had an increase in the variance of ratios in LF-E individuals (n = 5; 0.85 ± 0.473) when compared to LF-1 (n = 5; 0.83 ± 0.08). Peak 3 appeared only in the extracts of grasshoppers performing a long-duration flight.

LC-ESI-MS of differential HPLC peaks

Fractions containing peaks one and two were collected and their respective masses measured by LC-ESI-MS. The mass for peak 1 (MS 3702) and peak 2 (MS 3802) were similar to reported masses of a group of peptides belonging to the serine protease inhibitor family (Vanden Broeck et al.1998; Clynen et al. 2002;). These protease inhibitors act on a variety of protease cascade signaling and physiological systems ranging from initiation of pro-

phenoloxidase activation to mobilization of hemolymph amino acid reserves (Tong and Kanost, 2005; Telfer and Kunkel, 1991).

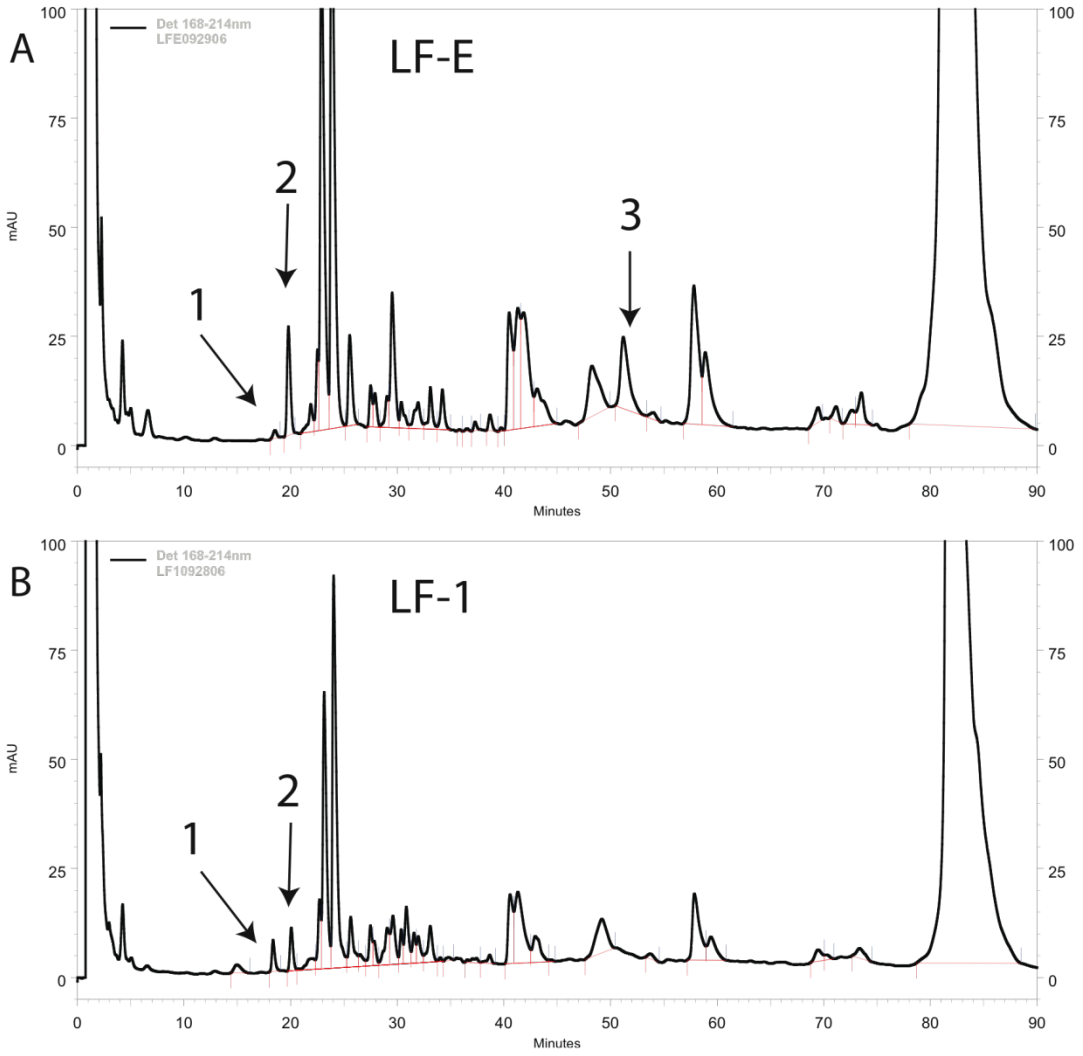


Figure 5.1: HPLC of LF-E vs LF-1 Hemolymph
Comparative HPLC 214_{nm} traces of *M. sagnuinipes* hemolymph extracts following performance of long-duration flight (A) or one-hour diagnostic flight (B). The ratio of peak one to peak two varied widely in grasshoppers who performed long duration flight (n = 5; 0.85 ±0.473) compared to those whose flight was terminated at one hour (n = 5; 0.83±0.08). Peak 3 was only present in extracts of those who performed long-duration flight and was tentatively identified as glycerol metabolites.

N- terminal sequencing and identification of differential peaks

The above peaks were determined likely to be serine protease inhibitors, characterized by the cysteine knot, by their mass signature (Fig. 5.2). It was necessary to ensure that any disulfide bonds were completely reduced before sequencing. Following this extensive reduction procedure we sequenced peaks 1 and 2 by N-terminal Edman degradation. Peak 1 was successfully sequenced for 21 cycles yielding a sequence of EQQCTPGQTKKEDCNNCTSGD. Protein-Protein Blast database query resulted in a match with the first half of the serine protease inhibitor precursor sequence of *S. gregaria* SGP-1 (E value: $3e^{-6}$ Swiss Prot: O46162). Peak 2 was sequenced for 36 cycles and produced a sequence of EISCEPGTTFQDKCNTCRCGKDGKSAAGCTLKACPQ. Protein-Protein Blast yielded a match with the second half of the sequence of the precursor for *S. gregaria* SGP-2 (E value: $4e^{-6}$; Swiss prot: O416162). These two peptides originate from a single precursor peptide known to inhibit trypsin (SGP-1) and chymotrypsin (SGP-2) respectively (Fig. 5.3) (Simonet et al., 2002).

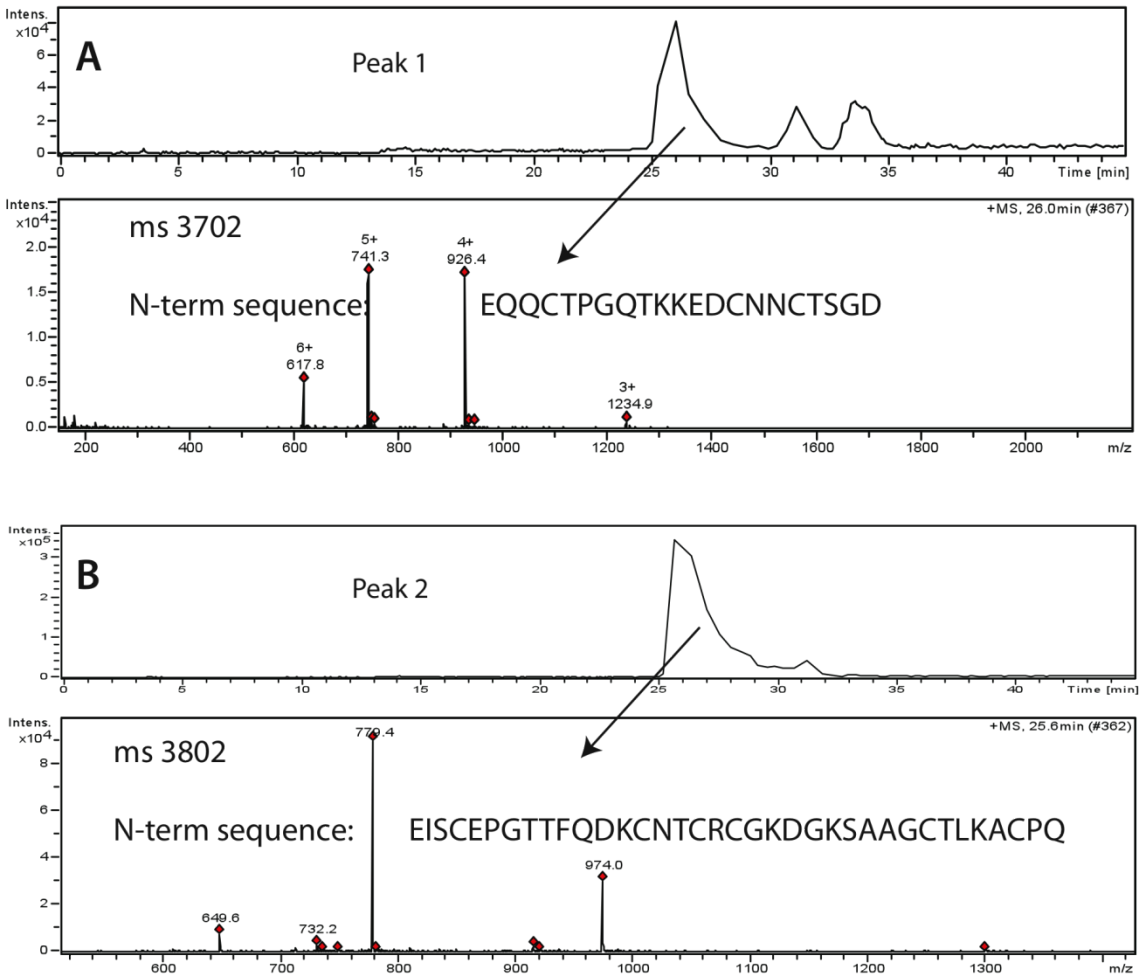


Figure 5.2: LC-MS of flight variable HPLC peaks.
LC-ESI-MS of variable HPLC trace peaks 1 and 2. Inset: N-terminal sequencing results for the separated peptides. Peak 1 was determined to have a mass of 3702 and its n-terminal sequence matched that of SGPI-1 of *Schistocerca gregaria*. Peak 2 had a measured mass of 3802 and its n-terminal sequence matched that of SGPI-2 of *Schistocerca gregaria*.

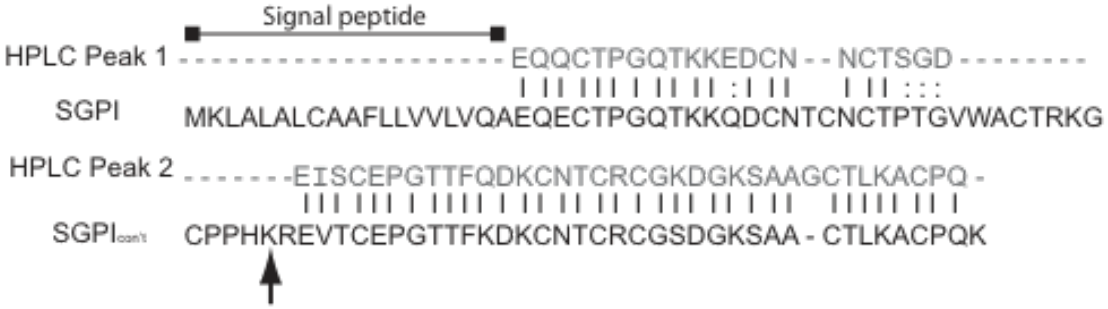


Figure 5.3: Sequence alignment of N-terminal SPI sequences

Sequence alignment of the n-terminal sequences obtained from HPLC peaks 1 and 2 with the serine protease inhibitor pre-cursor of *S. gregaria.* Arrow indicates the cleavage site that yields the two active peptides .

Discussion

Differences in the peptidome correlated with the performance of a long-duration flight were observed when compared to hemolymph from control animals that made only a one-hour flight or that did not fly. We found that the sequence data of the two peptides isolated from *M. sanguinipes* hemolymph showed amino acid homology with the serine protease inhibitors identified in *Locusta migratoria* and *Schistocerca gregaria*. They make up a family of peptides of 4 kDa serine protease inhibitors, stabilized by three intramolecular disulfide bridges (Gaspari, et al., 2002). These peptides, earlier designated as pasifastin light chain domains' (PLCDs), possess the conserved array of six cysteine residues (Cys-Xaa$_{9-12}$-Cys-Asn-Xaa-Cys-Xaa-Cys-Xaa$_{2-3}$-Gly-Xaa$_{3-4}$-Cys-Thr-Xaa$_3$-Cys) (Gettins, et al., 1996) and were shown in locust hemolymph extracts to inhibit proteolytic activation of the prophenoloxidase cascade, important in insect

immune defenses (Boigegrain, et al., 1992). Interestingly, five peptides with sequence homology have been isolated from ovarian tissue of the desert locust *S. gregaria* where they may play a role in the processing by serine proteases of vitellogenin into its crystalline form vitellin that is present in yolk (Hamdaoui et al., 1998; Tufail and Takeda, 2002).

Our qualitative analysis of the hemolymph peptidome from LF-E and LF-1 female *M. sanguinipes* revealed differences in the serine protease inhibitors. We found changes in peaks in LF-E versus LF-1 grasshoppers containing serine protease inhibiting peptides. The change in titer of the SPIs could reflect a selective activation or deactivation of the respective proteases they inhibit and may be an important part of the transformations that occur on lipophorin and vitellogenin after they pass the follicle cell epithelium of the oocyte and are packaged for storage.

The observed change in hemolymph serine protease inhibitors in response to long-duration flight may have some bearing on the prevention of stress-induced autoimmune response. The immune response of insects employs the HDLp-LDLp system to act as both a sensing component for the presence of foreign bodies and as a transporter for elements of the immune response (reviewed in Schmidt et al., 2008). In order to prevent the performance of flight from launching a potentially damaging autoimmune response these SPI changes might act to prevent the activation of the phenoloxidase immune response or the

conversion of lipid particles from transport to adhesive state. Both of these are dependant on serine protease cascades for their activation.

Understanding the hemolymph peptidome is important to understanding how flight performance, physiology and life history interact. The work presented here furthers our understanding of the complex physiological correlates of flight involving the hemolymph peptidome, and it enhances the methodological repertoire necessary to analyze it. As noted by Clynen et al. (2002) the lack of a fully sequenced orthopteran genome prevents the identification and subsequent physiological characterization of many of the variable components of chromatographic separations that may be importance. Once a fully sequenced and annotated orthopteran genome is available, it will be possible to utilize these methods to more completely examine how the multitude of putative peptides is correlated with the physiology of the insect's life history.

Chapter 6

Flight performance, survivorship and immune proteins

Introduction

The effect of exertion on the immune system is an important selective pressure in animals (reviewed in Pederson and Hoffman-Goetz, 2000). Migratory birds experience exertion on a scale unmatched in many species and have been shown to have greater immune reserves compared to closely related sedentary species (Moller and Erritzoe, 1998). In contrast, the highly migratory Red Knot *Calidris canutes*, displays no effect of long duration flight on the immune response (Hasselquist et al., 2007). *Homo sapiens* is the only primate in which performance of long duration physical exertion has evolved as a life history strategy (Lieberman and Bramble, 2007). In humans exertion has been shown to produce increased immunoglobin (IgG) levels and to induce potentially immune-enhancing IgG isotype switching (McKune et al., 2005). In insects the direct interaction of exertion and immunity is commonly thought to be a trade off between the demands of lipid transport for flight and the immune response (reviewed in Adamo, 2008). This tradeoff has been demonstrated immediately following flight in the cricket genus *Gryllus*. The cricket model has been used to examine the competition for resources between flight (fuel mobilization) and immune function during and immediately following short duration flight (Weers and Ryan, 2006, Adamo, 2008). What has not been investigated until now is

what these relationships are in a long distance migratory insect. *M. sanguinipes* has evolved physiological strategies to deal with the potential costs of long duration flight on reproduction (McAnelly and Rankin, 1986b; Burchstead, 1990; Min et al., 2004). Here we report results of an investigation into the effect of long duration flight on the immune response in male *M. sanguinipes* that could be important in surviving the various risks of migration.

During grasshopper flight one principal event that involves changes in the hemolymph is lipid mobilization by adipokinetic hormone (AKH) released from the corpora cardiaca to power the flight muscles (Arrese et al., 2001; Min et al., 2003). AKH initiates the signal cascade for the conversion of stored triacylglycerol (TAG) into diacylglycerol (DAG) for loading into HDLP at the fat body adipocyte hemolymph interface (reviewed in Arrese and Soulages, 2010). DAG is transported to the flight muscle by the lipophorin protein complex (Lp) (Fig. 6.1) where it serves as fuel for the flight muscles (Kent et al., 1997).

The action of AKH on the fat body is complex, and long-term exposure to AKH may have pleiotrophic effects (Reviewed in Arrese and Soulages, 2010). The response of fat body to AKH has been shown to vary between species,

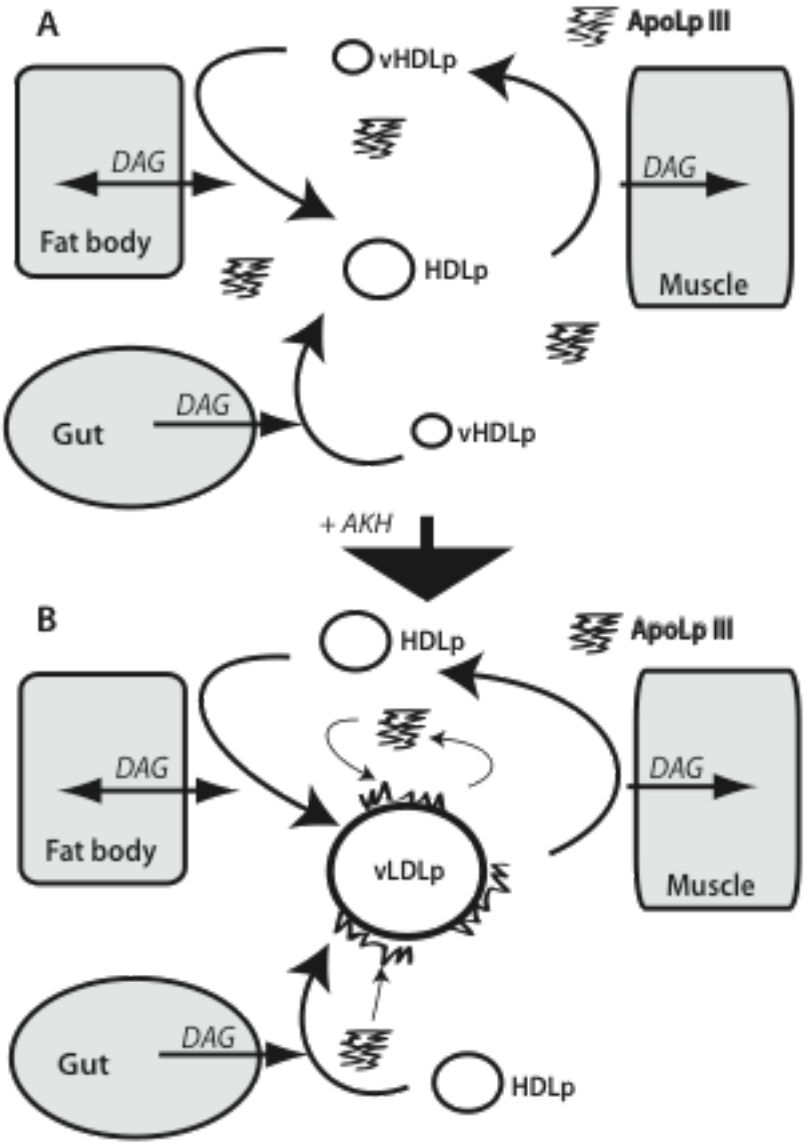


Figure 6.1: Lipophorin hemolymph dynamics
An illustration of the complex dynamics of the lipophorin transport system of grasshopper hemolymph during rest (A) and performance of flight (B). This lipoprotein system mobilizes and delivers diacylglycerol (DAG) to and from fat body, muscle and gut by the conversion of high density lipophorin (HDLP) into low density lipophorin (LDLP) through the incorporation of the exchangeable ApoLp III. This system also participates in the immune response as a sensory element and as a component of coagulation (Figure adapted from Adamo 2009)

according to whether or not migration is part of the life history strategy or the evolutionary history of the species in question (Zieglar et al., 1988; Min et al, 2003). AKH has also been shown to inhibit in vitro protein synthesis in locust fat body (Carlisle and Loughton, 1986; Moshitski and Applebaum, 1990).

In *M. sanguinipes* Min (et al., 2004) showed that AKH titers during flight reach a peak of 6 ng/ml during the first 60-120 minutes of flight. These titers then fall to a sustained level of 2 ng/ml for the remaining duration of the flight. Perhaps a difference exists in the AKH induced physiological effects of long versus short duration flights.

As reviewed in Chapter 1, Lp in the hemolymph exists between in a continuum between states, high density lipophorin (HDLp) and low density lipophorin (LDLp). These represent variation in the lipid loading state during the AKH-induced transport of fuel to the flight muscle (Fig. 6.1). Lp conversion between these two states is the result of conformation change of the exchangeable apolipohorin III (ApoLp III), which facilitates loading large amounts of hydrophobic diacylglycerol into HDLp to form LDLp (Ryan and Vanderhorst, 2000; Vanhoof et al., 2002; Soulages et al., 1996), all of which occurs at the fat body-lipophorin interface as a consequence of AKH release from the CC and its action on the fat body. Lp is a pleiotropic protein-lipid complex that plays a role in transporting DAG from the fat body to flight muscle during flight, from the gut to the fat body and from the fat body to the ovary during reproductive development.

Lp also participates in the cell free immune response (Zieglar and Antwerpen, 2006; Fan et al., 2002; Kawoaya and Lau, 1988).

In grasshoppers (and arthropods in general) the cell free immune response can be divided into two general mechanisms; sensors and effectors (Schmidt et al., 2008). Sensing involves identification of foreign bodies, whether they are bacterial, fungal, animal, or inorganic. This is accomplished by a wide range of extracellular sensor particles in the hemolymph such as Lp and peptidoglycan-binding proteins that attach to the foreign body (Schmidt et al., 2008; Leuller et al., 2003). Effector mechanisms are those that deal with a foreign body. Cell free effectors include the production of antimicrobial peptides, serine protease based pro-phenoloxidase activation, and encapsulation (Kanost and Gorman, 2008; Whitten et al., 2004). Some of the components of the immune response are observed in association with the Lp particle (Mullen and Goldsworthy, 2003; Schmidt et al., 2008). In the rat, knockouts of the gene encoding the homologous lipoprotein E are more vulnerable to bacterial infection than animals with the functional protein (De Bont et al., 1999; Laskowitz et al., 2000). Locusts form LDLp as part of their innate immune response when challenged (Goldsworthy et al., 2005; Dettloff et al., 2001). ApoLp III has a sensing immunological function via its capacity to bind lipopolysacharides (LPS) of bacteria. (Halawani and Dunphy, 1999; Weers and Ryan, 2006; Andrijkei et al., 2008). The LPS binding function of ApoLp III has been characterized as a component of the lipid binding moieties that are only exposed to the hemolymph

during its lipid bound conformation as a part of LDLp (Niere et al., 2001; Weers and Ryan, 2006).

Previous work has shown that *M. sanguinipes* females benefit reproductively from long duration flight. In this study a combination of methods was used to determine the effect of long duration flight on the immunological capacity of male *M. sanguinipes* and the grasshopper's ability to survive bacterial challenge. Given the pleiotrophic nature of Lp and its critical role during flight, understanding its full relationship to flight experience may yield insights into the costs and benefits of migration in this economically important insect. If hemolymph titers of Lp are affected by long duration flight experience, this may have a significant effect on survival of this colonizing insect as it enters new habitat patches and/or encounters physical injury and pathogens.

Methods

Experimental animals

As previously described (Kent et al., 1997) *M. sanguinipes* used for this study were first generation offspring of ~1000 individuals collected by sweep net on the San Carlos Apache Reservation east of Globe, Arizona.

Tethered flight assay

Grasshoppers were assayed using a tethered flight apparatus (McAnelly and Rankin, 1986). A small stick was attached to the pronotum of each grasshopper with wax, the grasshopper was suspended in front of a fan,

incandescent lights and an electric heater to simulate field conditions associated with migratory flights in this insect (Parker et al, 1955). Once flight was initiated animals in flight were monitored for continuing flight at least every 30 minutes. Flight times for animals found to have ceased flight between checks were rounded down to the nearest one half hour. For protein titer experiments, flights were terminated by the observer at various intervals. In the categorical challenge experiments individuals were migrants experiencing long duration flight to voluntary cessation (LF-E) or allowed to fly for only one hour (LF-1). This identified migrants but prevented them from actually performing a long duration flight.

Statistical analysis

Survivorship was analyzed by Kaplan-Meier estimator to determine the survival function. Spearman's correlation was used to analyze the relationship between protein titers and time of flight. Capillary electrophoresis titer data and phenoloxidase data were first analyzed by Pearson's product moment correlation. Secondary logistic regression analysis was performed on ApoLp I hexemerin and pro-phenoxidase activity using a sigmoid 4-parameter model. All calculations were performed in Sigma Plot (Sysstat, Chicago, Illinois) Statistical models were tested by residual analysis for normality.

Hemolymph collection and storage

Hemolymph was collected by puncturing the dorsal membrane between the head and pronotum with a sterile needle. For proteomic assays hemolymph

that flowed freely from the puncture (typically 5-10 µL) was collected by automatic pipette and diluted 1:10 with freshly prepared hemolymph protein isolation buffer (10mM Tris-HCl pH 8.0, 1µM PMSF, 1mM EDTA) and centrifuged at 4^{o}C for 5 minutes at 8000 RCF. One µL of reducing agent (10mM Dithrioreiotol or 150 mM 2-carboxyethyl phosphine) as added to the supernatant which was either analyzed immediately in the assays as described below or frozen at -80^{o}C for future analysis. For phenoloxidase assays hemolymph that flowed freely from the puncture (typically 5-10 µl) was collected by automatic pipette and diluted 1:20 with freshly prepared phosphate buffered saline (pH 7.5) and centrifuged at 4^{o}C for 5 minutes at 8000 RCF. The supernatant was collected and either analyzed immediately in the assays as described below or frozen at -20^{o}C for future analysis. Due to variation in hemolymph volume acquired from each grasshopper and variation in flight responses it was not possible to perform all assays on all samples.

Bacterial pathogen isolation and challenge

Three *M. sanguinipes* that had signs of bacterial infection were macerated, placed in 15 ml liquid broth (LB) media (5 g/l tryptone, 2.5 g/l yeast extract, 1 g/l NaCl pH 7.5) and incubated in a rotary shaker over night at 30^{o}C. The resulting mixed bacterial culture was then serially plated on LB agar plates and incubated at 30^{o}C over night. Four different colonies were identified by plaque morphology and color. These four colonies were used to inoculate fresh enriched LB media

and incubated at 30^{o}C overnight. Their respective optical densities were measured at 600_{nm}, and each culture was diluted with sterile LB to an optical density of 0.1 OD 600_{nm}. Male grasshoppers (n=4 each isolate) were injected with 1 µl of the four bacterial isolate dilutions between the second and third abdominal body sternites with a sterile gauge 701 Hamilton syringe. Isolate number 3 was determined to be pathogenic (no mortality was observed in the other three isolates) and used in all subsequent bacterial challenge experiments. Isolate #3 was again cultured over night and 5% glycerol v/v frozen stock cultures were prepared and maintained at –80^{o}C. All subsequent experiments were conducted with fresh overnight cultures.

Genomic DNA was purified from bacterial isolate #3 by the Wizard gDNA purification kit (Promega). Ribosomal DNA was amplified by PCR following the protocol of Christenson (et al. 2005). Primers were obtained from Integrated DNA Technologies (Forward 5' AGAGTTTGATCCTGGCTCAG 3' Reverse 5' ATTACCGCGGCTGCTGGC 3'). PCR product was purified with the Qiaquick spin kit (Qiagen Sciences). Sequencing of an ~400 BP product at the UT DNA core facility was successful. Blast search optimized for highly similar sequences (megablast) against the nucleotide collection (nr/nt) resulted in a 97% identity match (e value 0.0) with the 16s ribosomal RNA gene of the gram negative bacteria *Klebsiella oxytoca* (NCBI:GU993916.1). *K. oxytoca* is a known insect pathogen and an opportunistic BSL-2 pathogen of humans (Lysenko, 1985). All subsequent experiments were conducted within BSL-2 regulations.

We tested the effect of flight experience on male *M. sanguinipes'* capacity to survive an immune challenge. Male and female grasshoppers were assayed for flight and separated into diagnostic migrants (LF-1; Male n = 14 Female n = 8) or allowed to fly to voluntary cessation (LF-E; Male n = 14 Female n = 8) on day 4 post eclosion. On day 5 each group was challenged with 0.1 µl of a fresh 0.2 OD 600_{nm} *K. oxytoca* culture as described above. Grasshoppers were observed for survivorship for 30 days. Control male and female grasshoppers (N = 7 per sex) were injected with 0.1 µl of sterile LB on day 5 post eclosion. No mortality was observed in these grasshoppers.

Hemolymph proteomics

We determined total protein titers by the colorimetric Bradford assay (Bio-Rad) with bovine serum albumin as the standard. Absorbance was measured on a Shimadzu UV-1201 UV-Vis spectrophotometer at 595_{nm}.

Cell free hemolymph was prepared as described above to separate the protein components of *M. sanguinipes*. We separated proteins by 4-12% gradient SDS-PAGE (NuPage Invitrogen) with TECP as the reducing agent following the manufacturers instructions. Electrophoresis was performed at constant voltage (120 V) on ice to prevent streaking. Gels were stained in coomasie blue, destained and 7 prominent protein bands were isolated for protein identification. We blotted the band suggested to be ApoLp III to a PVDF membrane and excised it. The band was loaded into the reaction chamber of an Applied

Biosystems Procise 492clc protein sequencer for N-terminal sequencing via Edman-degradation. 23 sequencing cycles were completed.

Seven prominent proteins were submitted for identification in collaboration with the Protein Micro-facility of the Institute for Cellular and Molecular Biology University of Texas. For MALDI-TOF SDS-PAGE gel bands were dehydrated in acetonitrile, reduced with 10mM DTT, alkylated in 50mM iodacetamide, washed with 100mM ammonium bicarbonate and dehydrated again in acetonitrile. Digestions were performed with 10µg/ml trypsin in 50mM ammonium bicarbonate, incubated overnight and extracted the next morning with 5% formic acid in HPLC grade water. Protein identities and associated e-values were determined by probability-based MOWSE score against the NCBI, SwissProt and Trembl protein sequence databases using the software suit of the Applied Biosystems 4700 Proteomic Analyzer. The threshold for database hit significance was 0.05.

Apolipophorin I, hexemerin and apolipophorin III titers were determined by an Agilent (APB PC-230) capillary electrophoresis system at the UT Protein Facility (Figure 3). Protein size and relative quantity were determined by retention time and fluorescence peak area respectively. Manufacturer's directions were followed with regard to incubations and dilutions with the exception that a thermocycler and 0.1 ml PCR tubes were used for the 95^{o}C denaturation step (95 ^{o}C for 5 minutes then 4 ^{o}C $\propto$). This provided better peak resolution than the recommended 95 ^{o}C water bath. Relative quantities were calculated using the

manufacturers internal standard. These measurements are referred to as relative due to the manufacturers warning of variation in staining intensity for different proteins. Lp found in the hemolymph is produced from the post-translational modification of a single large precursor protein originating in the fat body into the two apoprotein structural components apolipophorin I (~250 KD) and II (~70 KD) (Weers et al., 1993). We report Lp titers determined by the peak area of the larger apoprotein component ApoLp I.

Phenoloxidase activity is commonly used as an index for the measurement of the immune activation state of insects and pro-phenoloxidase as the immune reserves available to a given insect (Goldsworthy et al., 2005). This distinction is a result of the majority of phenoloxidase being present in the hemolymph as an inactive multi protein zymogen awaiting conversion into active enzyme by the action of a serine protease cascade (reviewed in Ragan et al., 2009). Active phenoloxidase was determined by the increase in absorbance at 492_{nm} of a 0.3 mg/ml dopamine solution following addition of cell free hemolymph using the kinetics function of a Beckman Coulter (Model DTX 880) plate reader following the protocol of Mullen and Goldsworthy (2003). Pro-phenoloxidase was converted from inactive zymogen to active enzyme by reaction with methanol and its activity measured using the same protocol described above. Results are expressed as change in absorbance at 492nm / minute / µl hemolymph.

Results

Flight performance and survival of a bacterial challenge

Genomic DNA was purified from bacterial isolate #3 and diagnostic 16s ribosomal DNA was amplified by PCR following the methods of Christenson et al. (2005). Sequencing of a 400 BP region was performed at the UT DNA facility. Blast search resulted in a score of 719 and 96% identity match with the 16s ribosomal DNA of the gram negative bacterium *Klebsiella oxytoca (e = 0)*. This insect pathogen is an opportunistic pathogen of humans and is considered a biosafety level 2 organism.

We used Kaplan-Meier to analyze the effect of flight to voluntary cessation on the survival function of LF-1 vs LF-E male and female *M. sanguinipes* challenged with *Klebsiella oxytoca*. LF-E males had a mean survival time of 17.43 days (log-rank Chi^2 = 8.58925; P = 0.003) significantly higher than diagnostic LF-1 migrants who had a mean survival time of 4.43 days (Fig 3.2 a).

In females the experience of flight to exhaustion (LF-E) did not confer enhanced survivorship after challenge with *K. oxytoca* relative to LF-1 animals (log-rank Chi^2 = 1.25827; P = 0.533) (Fig 6.2 b). It is possible that the lack of increased survivorship in females may be a result of rapid sequestration of lipophorin by the ovary during oogenesis, which we know is accelerated in *M. sanguinipes* females following long duration flight (Min et al., 2004). Control indivudals of each sex (N = 7) injected with sterile LB survived the full length of the study.

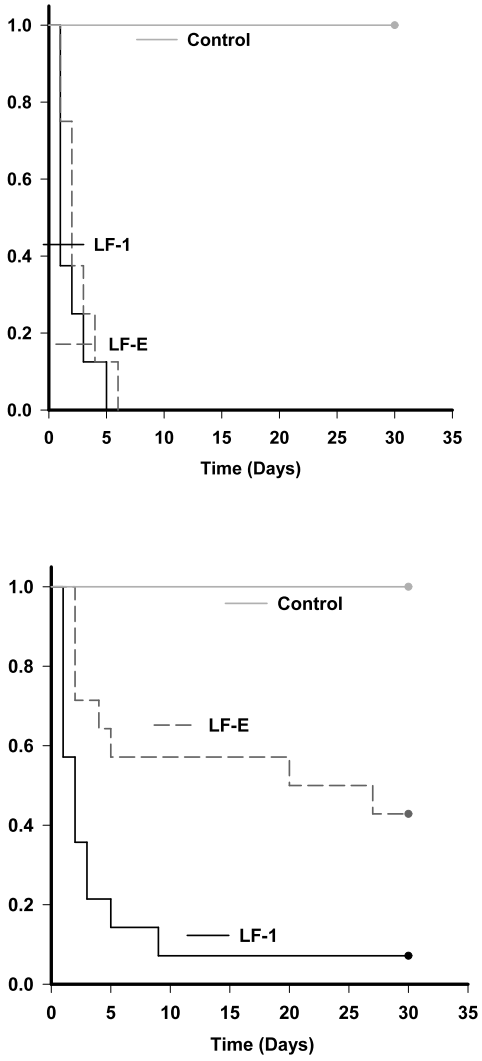


Figure 6.2: Flight performance and bacterial challenge survivorship.
Survivorship for individuals experiencing long duration flight versus those experiencing 1 hour diagnostic flight. Male *M. sangunipes* who performed long duration flight challenged with *K. oxytoca* had a significantly higher probability of survival (b) than those whose flights were terminated at one hour. Interestingly no difference in survival probability was observed in females (a) experiencing long duration flight versus controls. LF-E males (n = 14) had a mean survival time of 17.43 days (log-rank Chi^2 = 8.58925; P = 0.003) significantly higher than diagnostic LF-1 migrants (n = 14) who had a mean survival time of 4.43 days. Female LF-E *M. sanguinipes* (n = 7) survival versus diagnostis LF-1 migrants (n = 7) was unchanged (log-rank Chi^2 = 1.25827; P = 0.533). Control individuals (N = 7 each sex) injected with sterile LB broth showed no mortality during the 30 day observation period.

Effect of flight performance on hemolymph proteomics

Total hemolymph protein measured in *M. sanguinipes* 24 hours after 1, 4 or 7 hours long duration flight was directly correlated with duration of flight (Fig 6.3) Spearman's Rho = 0.7904; df = 13 p=0.0008).

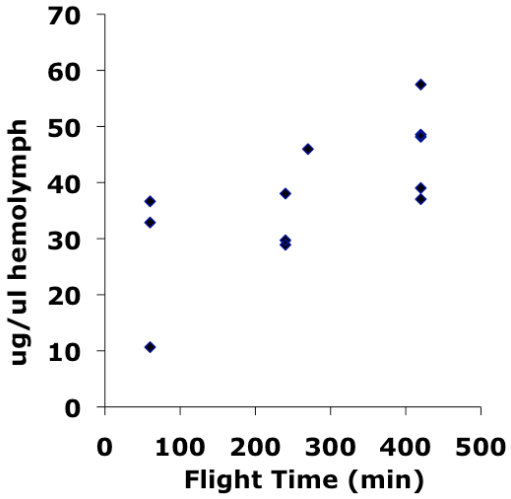


Figure 6.3 Total hemolymph protein titers. Total male hemolymph protein titers 24 hours following experience of 1, 4 or 7 hours long duration flight was directly correlated with duration of flight (Spearman's Rho = 0.7904; df = 13 p=0.0008).

Four of the predominant components of the hemolymph proteome were identified by a combination of analytic methods (Fig. 6.4). Three proteins, hexamerin, transferrin and apolipoprotein I were identified by in gel tryptic digestion, MALDI-TOF-TOF and probability-based Mowse scoring as follows: 1) the storage protein hexamerin had a P-score of 163, Total Ion Score of 155, a database match of GI:60256959 and an E value of $9.3e^{-11}$; 2) The iron transporter transferrin had a P-score of 373, a Total Ion Score of 334, a database match of GI:59939802, and an E value of $7.4e^{-74}$; 3) apolipoprotein I had a P-score of 174, a Total Ion Score of 150, a database match of GI:152031559, and an E value of $2.9e^{-26}$. Hexamerin is reported to function in the hemolymph as an amino acid storage reservoir for protein synthesis in the fat body (Hathaway et al., 2009). Transferrin is an iron transporter and may play some role in the immune response (Yoshiga et al., 1997). Apolipophorin I is the larger of the two structural apoprotein components of lipophorin. The fourth hemolymph protein identification was done by sequencing a 23 amino acid sequence (N-term:

DAAQVPDFATAVQNLxHTLSEAA) by N-terminal edman degredation, and this was identified as the exchangeable ApoLp III by protein blast (Swiss prot Q7M480; E value = $2e^{-9}$).

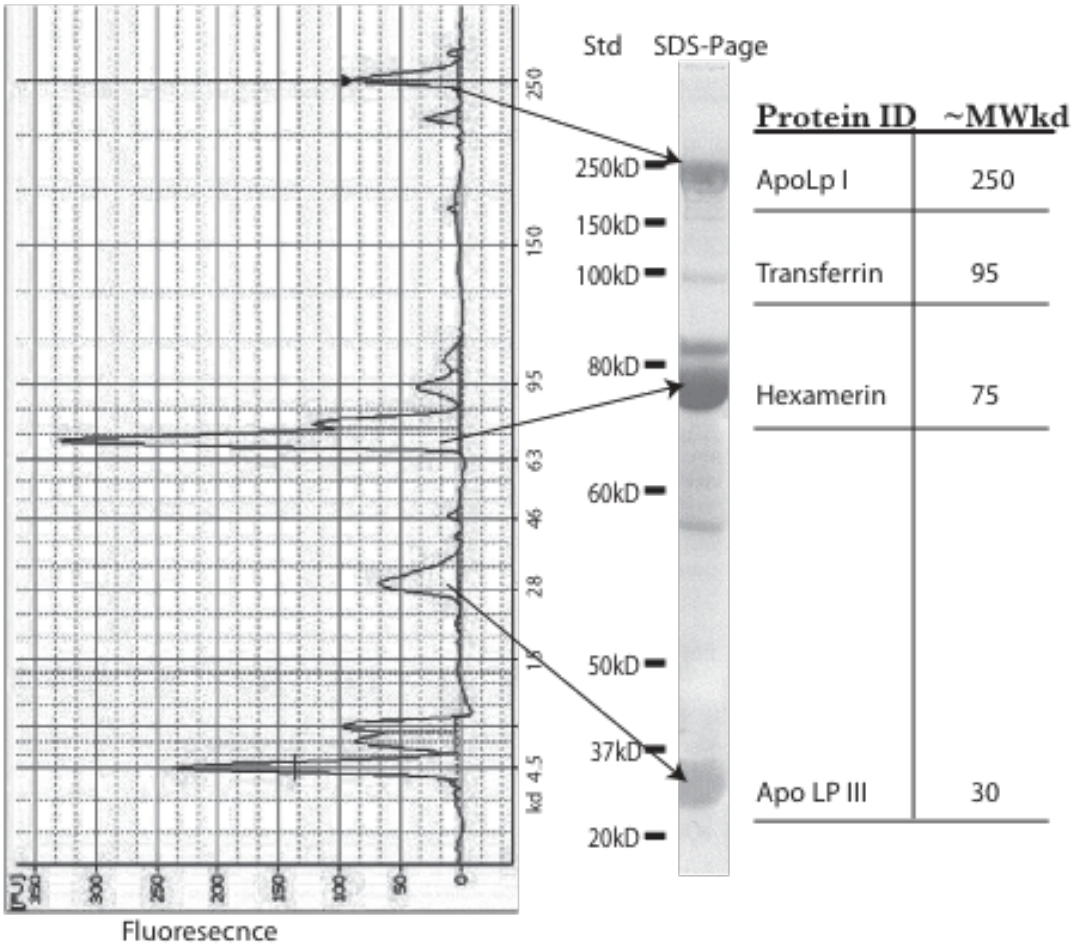


Figure 6.4 Proteomics of *M. sanguinipes* hemolymph.
A: Correspondence of representative electropherogram to SDS-PAGE bands identified by either N-terminal or proteolytic digestion and MALDI-TOF-TOF anlaysis. The horizontal and vetical axis on the electropherogram are fluorescence intensity and molecular weight respectively.

Linear regression model

Pearson's product moment correlation was used to examine the correlation of Hex, ApoLp I and ApoLp III titers with the duration of flight (Fig 6.5 A-C). The exchangeable ApoLp III did not show a significant rise in titer correlated with flight time ($r = 0.186$; $n = 23$; $p = 0.374$). Lp titers were positively correlated with duration of flight ($r = 0.639$; $n = 23$; $p = 0.001$) as was Hex ($r = 0.643$; $n = 23$; $p = 0.0009$). Lp and Hex both transit into and out of the fat body while ApoLp III is found exclusively in the hemolymph (Van Hoof et al., 2002; Hathaway et al., 2009). This suggests that the effect of flight duration may manifest at the lipid droplet Lp-hemolymph interface more dynamically than previously thought. ApoLp I, ApoLP III and Hex titers (Fig 6.5 D-F) were also measured in a smaller set of female grasshoppers ($N = 7$). No correlation between flight duration and titers was observed (ApoLp I: $r = 0.0397$; $n = 7$; $p = 0.933$) (ApoLp III rho = 0.131; df = 6; $p = 0.780$) (Hex: $r = 0.009$; $n = 7$; $p = 0.984$).

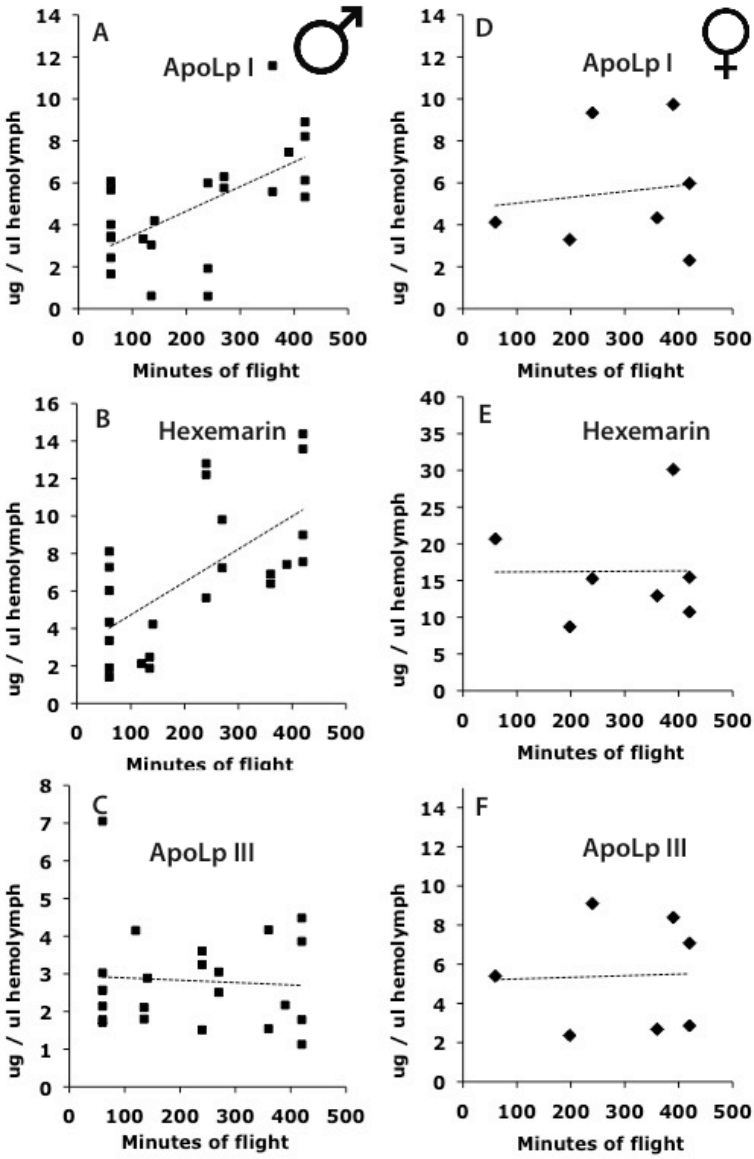


Figure 6.5: Hemolymph protein titers.
Capillary electrophoresis titers of hemolymph proteins correlated with time of flight. (A) ApoLp I titers were positively correlated with duration of flight (r = 0.639; n = 22; p = 0.001). (B) The hemolymph storage protein hexemerin also was correlated with the time of flight (r = 0.643; n = 22; p = 0.0009). (C) The exchangeable ApoLp III did not show a significant rise in titer correlated with time flight (r = 0.186; n = 22; p = 0.374). (D-F) No correlation between flight duration and titers was observed in female *M. sanguinipes* (ApoLp I: r = 0.0397; n = 7; p = 0.933) (ApoLp III r = 0.131; n = 7; p = 0.780) (Hex: r = 0.009; n = 7; p = 0.984).

Pearson's product moment correlation was used to examine how activity of the immune response enzyme phenoloxidase and its inactive zymogen pro-phenoloxidase were correlated with flight duration (Fig 6.6). The activity of phenoloxidase was not correlated with time of flight ($r = 0.596$; $n = 10$; $p = 0.069$). The activity of the pro-phenoloxidase zymogen ($r = 0.763$; $n = 11$; $p= 0.006$) was increased in correlation with the duration of flight. This increase in the pro-phenoloxidase immune reserve reflected the general observation of increased total hemolymph protein titers in *M. sanguinipes* in correlation with the duration of flight.

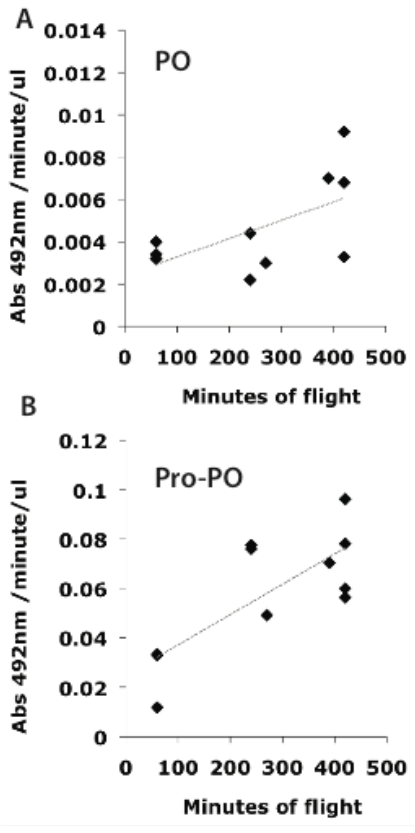


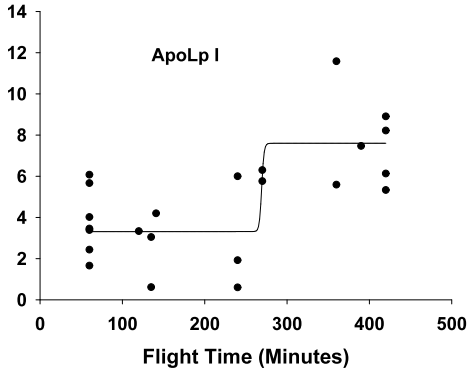
Figure 6.6: Phenoloxidase activity. Correlation of the activity of the immune enzyme phenoloxidase in male grasshoppers with time of flight (A: $r = 0.565$; $n = 10$; $p = 0.069$) as well as it's inactive zymogen pro-phenoloxidase (B: $r = 0.590$; $n = 11$; $p = 0.006$).

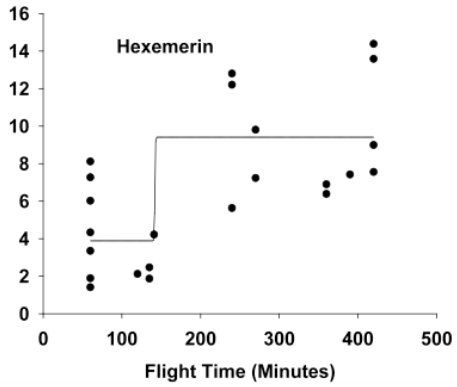
Logistic regression model

A linear correlation provided a statically significant model for the interaction of flight and hemolymph protein titers (Fig 6.5). Where correlation was found between flight time and hemolymph protein titer an alternate correlation using nonlinear regression was explored for whether the relationship might be better modeled as a threshold trait. We used a sigmoid 4-parameter logistic model with flight as the independent variable and titers as the dependent variable (Fig 6.7) apoLp I titers (R^2 = 0.5548; f = 7.8926, df = 22; P = 0.0013) hexemerin titers (R^2 = 0.5241; f = 6.9737; df = 22; P = 0.0024) and pro-phenoloxidase activity (R^2 = 0.6975; f = 5.379; df = 10; P = 0.03). The logistic model predicts a higher proportion of the variation of titers when compared with the linear model (Table 6.1).

	Logistic Regression		**Linear Regression**	
	r^2	P	r^2	P
ApoLp I N = 23	0.55	0.0013	0.41	0.001
Hex N = 23	0.52	0.0024	0.41	0.0009
Pro-PO N = 11	0.70	0.0310	0.35	0.006

Table 6.1: Comparison of linear versus logistic regression.
Comparison of measures of correlation and the associated P values from linear (Fig 6.5) versus logistic regression models (Fig 6.7) of the relationship of time of flight with hemolymph titers of ApoLP I, Hexemerin and Pro-PO.





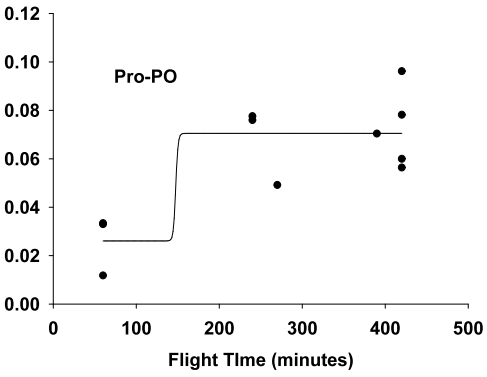


Figure 6.7: Logistic regression.
Four parameter sigmoid logistic regression models of ApoLp I titers (R^2 = 0.5548; f = 7.8926, df = 22; P = 0.0013) hexemerin titers (R^2 =0.5241; f = 6.9737; df = 22; P = 0.0024) and Pro-phenoloxidase activity (R^2 =0.6975; f = 5.379; df = 10; P = 0.031) with time of flight. The logistic regression models suggests that the enhancement of immunity following flight is a threshold trait that requires at least 120 minutes of flight to be initiated and perhaps as much as 280 minutes of flight.

Discussion

Male *M. sanguinipes* that have performed a long duration flight are more likely to survive a challenge from infection with the bacterium *Klebsiella oxytoca* than animals that have been identified as migrants but not allowed to fly to voluntary cessation. Furthermore when we performed a proteomic analysis of the predominant hemolymph proteins of male *M. sanguinipes* that performed flight of various durations, we found an increase in total hemolymph protein and more specifically in the lipid transport particle structural protein Lp, the immune enzyme phenoloxidase, and its inactive zymogen pro-phenoloxidase activity. Females on the other hand show neither increased resistance to infections nor increased protein titer after long duration flight. This may be because Lp is sequestered by the oocytes during oogenesis (Sun et al., 2000). Indeed Lp can make up a significant percentage of the total lipoprotein content found in the yolk (Kawooya et al., 1988). Long duration flight in *M. sanguinipes* results in acceleration of oogenesis and may cause rapid depletion of any build up of Lp from the hemolymph resulting from flight performance. The male-specific correlation between Lp titers and survival of infection after flight suggest that Lp may be a causative agent for the observed increase in survivorship in male *M. sanguinipes* that was not observed in females performing the same flights.

The interaction of flight experience and immunity is integral to understanding the broader context and evolution of a migratory life history. These interactions often manifest themselves during the reproductive phase of the

migrating insect's general life history (Dingle, 1972; Dingle, 1996; Min et al., 2004; Maeno and Tanaka, 2008). The experience of flight alters reproductive output negatively in *Anthonomus grandis* (Rankin et al., 1994) or positively in *M. sanguinipes* (McAnelly and Rankin, 1986; Burchstead, 1990; Min et al., 2004). How experience of flight interfaces with the Lp system also varies significantly among orders of insects and even between phyla (Zieglar et al., 1988; Auerswald and Gade, 1994). Similarly, understanding variation in the relationship between physiology and immunity has proven confusing and sometimes contradictory (Adamo et al., 2001; Goldsworthy et al., 2006; Dhabhar, 2009). It is important to consider the evolutionary life history trajectory of the insect being studied as well as the overlapping physiological networks and the individual variation in duration and intensity of flight experience. For an insect in which flight is used primarily as a means of foraging or escaping over short-distances it is unlikely that selection would favor a suite of physiological adaptations dependent on performance of long duration flight. For an insect such as *M. sanguinipes* that uses long distance migration as an integral part of its colonization strategy, the selective advantage of flight-induced immune investment is obvious.

The intercellular lipid droplet of adipocyte fat body cells is a dynamic organelle enveloped in protein not a simple lipid storage bundle. Similarly the Lp transport particle of the hemolymph may have numerous important physiological functions including resistance to infection (reviewed in Aresse and Soulages, 2010). Following receptor-mediated endocytosis Lp follows a transferrin like

intercellular cycling pathway as it transits into the adipocyte fat body cell where it interacts with the lipid droplet (Van Hoof et al., 2002). What happens next at the lipid droplet-Lp interface is poorly understood (Fig. 6.8 reviewed in Aresse and Soulages, 2010).

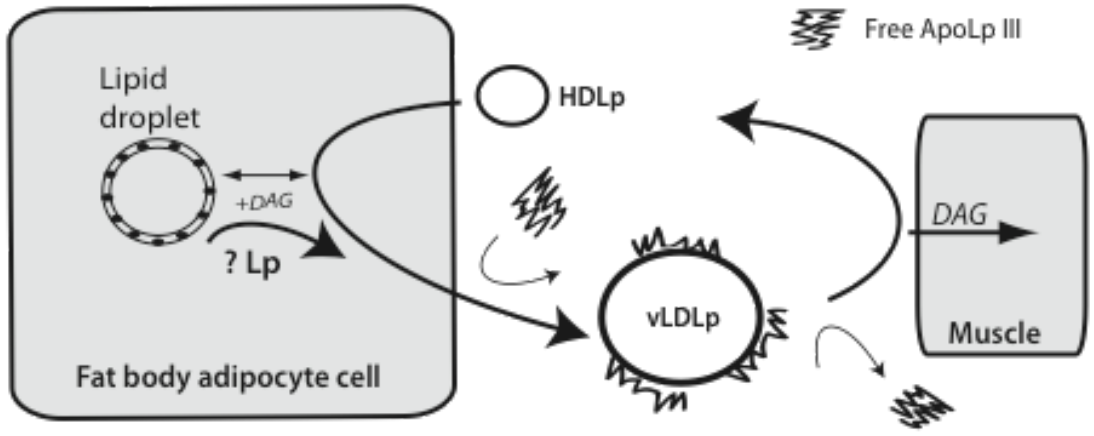


Figure 6.8: Lp, AKH and the fat body.
Hypothetical model of the interaction of Lp, AKH and the fat body lipid droplet. Prolonged exposure to steady state titers of AKH may promote the mobilization of reserves of Lp in addition to the DAG that is normally loaded at the fat body hemolymph interface.

It is likely that the adipocyte lipid droplet / hemolymph interface is more complex than current models predict. Given the observed increase of proteins (ApoLp I and Hexemerin) that originate in the fat body, perhaps the long-term exposure of the fat body to AKH mobilizes reserves of Lp into the hemolymph. Min (et al. 2003) observed that AKH titers are high during the first 90 minutes of flight then drop to ~2ng/μL for the remainder of flight duration. When considered with the Lp, Hex and Pro-PO logistic regression model data (Fig 6.7) presented here, it is apparent that the relationship between AKH titer during flight and the homeostatic change in Lp titer is important (Fig. 6.9). There appears to be an inverse

relationship between the AKH titers during flight and the amount of residual lipophorin and arylphorins that builds up in the hemolymph reaching homeostasis at between 120 and 250 minutes. Further research on the adipocyte lipid droplet will help to understand how increased circulating titers of Lp and not ApoLp III might result from the experience of long duration flight in male *M. sanguinipes.*

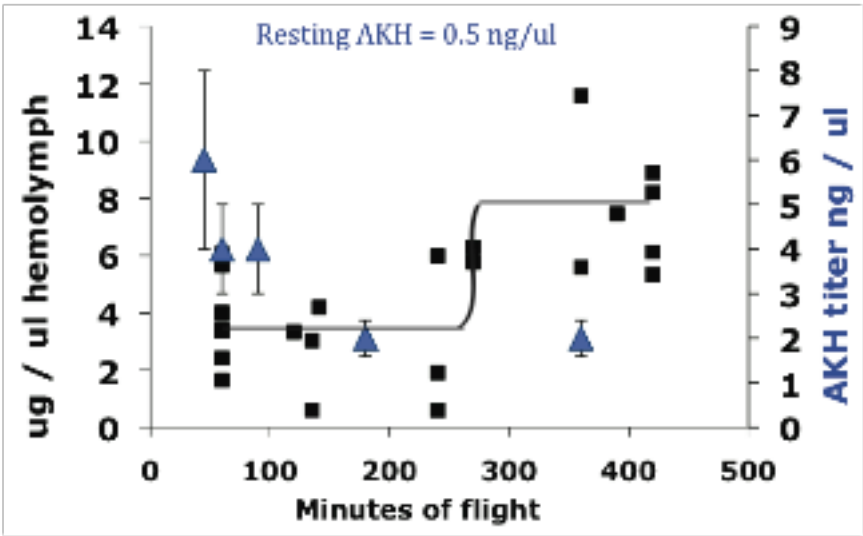


Figure 6.9: Lp titers, AKH and flight duration.
Lp titers plotted with AKH titers against the duration of flight performance. As AKH titers reach a steady state at 90 minutes the titer of Lp present in the hemolymph begins to rise. (AKH data used with permission from Min et al, 2003).

The work described here is the first to examine the effect of duration of flight on hemolymph Lp titers following long duration flight. The 24-hour interval between flight experience and the various assays conducted means that we were examining the long term rather than the immediate impact of flight experience. The capacity of a migrant to survive in a novel habitat is closely linked with its immune function (Kurtz et al., 2002). The work presented here shows that

following experience of long duration flight, male *M. sanguinipes* have an increase in survivorship when challenged with the bacterial pathogen *Klebsiella oxytoca*. The increase in survivorship is correlated with an increase in immune related protein titers. It is possible that other unobserved factors related to flight are responsible for the increased survivorship. However given the established role for lipophorin and phenoloxidase in the immune response of orthopteran insects, our results suggest that these proteins play a major role in the increased survivorship observed following long duration flight. Locusts disease resistance has also been shown to be higher in gregarious versus solitary phase animals (Wilson et al., 2002). While locust swarming does not typically include the same long-duration individual flights observed in *M. sanguinipes*, it does involve long distance movement via many short individual flights (Johnson, 1969). *M. sanguinipes* is found over most of North America (Capinera et al., 2004). Populations of *M. sanguinipes* also vary widely in their respective migratory disposition, morphology and life history (McAnelly, 1984). This suggests that the observed relationship between migratory experience and immunity may vary even within a species across geography.

Together these data indicate that the hemolymph titers of Lp and PO increase with the duration of flight experience and that these titer changes may contribute to a significant change in the immune function and observed increase in survivorship of male *M. sanguinipes* experiencing long duration flights. The work presented illustrates how complex the interaction of long duration flight and

hemolymph proteins may be, and it provides a potential target system for future efforts to design and effect control strategies.

Chapter 7

Conclusions

This study examined the impact of flight experience on the immune capacity, hemolymph protein physiology and the effect of perturbations of JH on the follicular epithelium of the migratory grasshopper *M. sanguinipes*. It began under the following general hypotheses that:

1: long duration flight experience causes female *M. sanguinipes* to increase their food intake, digestive efficiency, mating frequency or duration as compensation for the resource costs of flight in *M. sanguinipes*.

2: the physiology, morphology, and relationship of reproduction to flight performance vary in different populations of *M. sanguinipes*.

3: early life exposure to JH III alters the sensitivity of ovarian tissues to *in vitro* JH III incubation.

4: flight experience impacts the immune function and hemolymph physiology of *M. sanguinipes*.

5: flight duration has an impact on the circulating titer of the lipid transport proteins of the hemolymph, specifically increasing titers of the exchangeable lipoprotein ApoLp III.

Female *M. sanguinipes* were repeatedly measured for the presence of compensatory feeding, digestion, mating frequency and copulation duration

following performance of long duration flight. A significant difference was observed in the weight gain of females who performed long flights, but no difference was observed in the ingestion or digestion of food or of the frequency or duration of copulation. It is clear that complex and subtle physiological changes occur following performance of long-duration flight that are still incompletely understood in spite of the fact that they have profound implications for survival and reproductive success.

McAnelly (1988) demonstrated intrapopulation variability in migratory propensity. We continued this line of inquiry with a comparison of morphometrics and physiology of *M. sanguinipes* populations from Arizona and Colorado. The Arizona population that has long been the subject of research into flight enhanced reproduction was found to be significantly larger, possess more ovarioles per ovary and to enter diapuse at a different state than the population derived from Colorado. Interestingly the population from Colorado also did not display the reduced time to first oviposition in response to long duration flight experience. This discovery will help to further our understanding of the evolution of this powerful life history trait in a colonizing insect.

Min (et al, 2004) demonstrated that JH III treatment is sufficient to recapitulate the reduced time to oviposition that is observed in female *M. sanguinipes* following long duration flight performance. The follicle cell epithelium plays an important and largely JH III-dependant role in the physiological architecture of oocyte maturation, through its role in patency and vitellogenin

incorporation to the developing oocyte. Following the establishment and validation of an in vitro patency assay utilizing filamentous actin, the effect of early adult life exposure to JH III was tested for its impact on subsequent in vitro incubation of oocytes with JH III and the level of follicle cell epithelial patency induced. JH III treatment reduced the threshold for induction of patency from 10^{-5} M JH III to 10^{-7} M JH III. This discovery furthers our understanding of the regulatory steps involved in oogenesis as well as providing a framework within which to understand the mechanism by which a short burst of JH can act to reduce the threshold for patency and possibly long-term fecundity.

This study has shown that long duration flight experience has a profound influence on some aspects of the physiology of *M. sanguinipes*. Although no neuropeptides were identified, flight had the effect of altering the ratios of small serine protease inhibiting peptides following long duration flight. It is unclear what role if any these peptides may play in the phenomenon of flight enhanced reproduction but the influence of flight on their titers may provide clues to the proteomic network underlying it.

Upon isolation and identification of the insect pathogen *Kliebsiella oxytoca* *M. sanguinipes* were shown to display a sexually dimorphic immunological benefit from long duration flight performance. Males that performed these flights had a higher probability of survival of a *K. oxytoca* challenge than control males. Females in contrast showed no change in the probability of survival following the bacterial challenge.

Flight duration was considered for its impact on the circulating titers of hemolymph proteins. The major proteins of the hemolymph were identified by a combination of SDS-PAGE analysis, mass spectrometry of SDS-PAGE proteolytic fragments, N-terminal sequencing and bioinformatics. The hemolymph proteins apolipophorin I, apolipophorin III, hexamerin and transferrin were successfully identified. Their respective titers were determined by capillary electrophoresis of hemolymph of grasshoppers performing variable length long duration flights. The hypothesis that ApoLp III titers were increased in correlation WITH flight duration proved to be incorrect. ApoLp I however was positively correlated with the duration of flight. Interestingly the hemolymph storage protein hexamerin was also positively correlated with the duration of flight. When the titers that were positively correlated with flight time were subjected to logistical regression analysis it was discovered that a sigmoidal relationship existed between flight time and the titers of ApoLp I, Hexemerin and Pro-phenoloxidase. The curves indicate that a threshold for the induction of the potentially immune enhancing titer increases exists between 120 and 240 minutes of flight.

This work showed that the impact of flight is not limited to the reduction of latency to first oviposition in female *M. sanguinipes* but also extends to the enhancement of immune function in male *M. sanguinipes.*

Appendix 1

Pathogens of *M. Sanguinipes*

Introduction

The predators, parasites and pathogens of *Melanoplus sanguinipes* occupy almost every order of the insect kingdom. Some of these threats are of anthropogenic origin and have become endemic in the field. I focus here primarily on pathogens that have an impact on either the migratory life history evolution of *M. sanguinipes* or the laboratory rearing of an annual field-derived F1 generation capable of producing individuals willing to display long-duration flight.

Insect Parasitoids

The most interesting parasitoid of *M. sanguinipes* is the sarcophagid genus *Blaesoxipha* (Sherwell, 1987). These common Dipteran parasitoids are larviparous. That is, the eggs develop into larvae while still within the female reproductive system. The larvae are deposited on potential hosts by a specialized ovipositor during aerial encounters (Personal observation; Danyk et al., 2003). Grasshoppers in the field performing flights are pursued by as many as three or more flies at a time in areas of high grasshopper density (Personal observation). Flies made numerous oviposition attempts on the abdomens of grasshoppers during flight. Grasshoppers were observed to actively evade the flies during these oviposition attempts (Personal observation). Upon successful oviposition the larvae begins to burrow through the cuticle, and the host attempts to encapsulate the larvae (Fig. A.1).

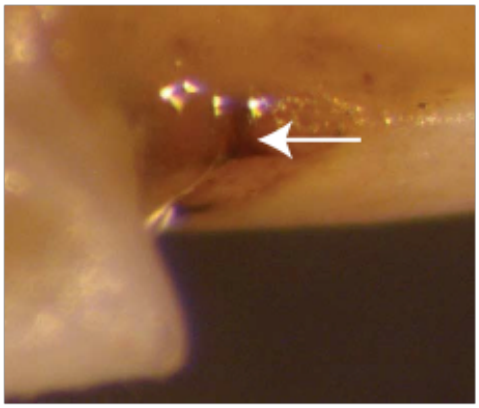


Figure A.1: *Blaesoxpha* larval entry site. Site of larval entry and discoloration of the clotting response. The scleritized respiratory tube illustrated in Fig. 2. begins here on the interior abdominal wall.

Upon overcoming the host's immune response the larva begins to grow, moving farther into the body cavity and maintaining a scleritized tube-like structure leading from its posterior end to the cuticle of the host grasshopper. This tube likely provides a means for respiration and excretion (Fig. 6.2). Upon reaching maturity the larva releases from it's anchor tube and burrows to the

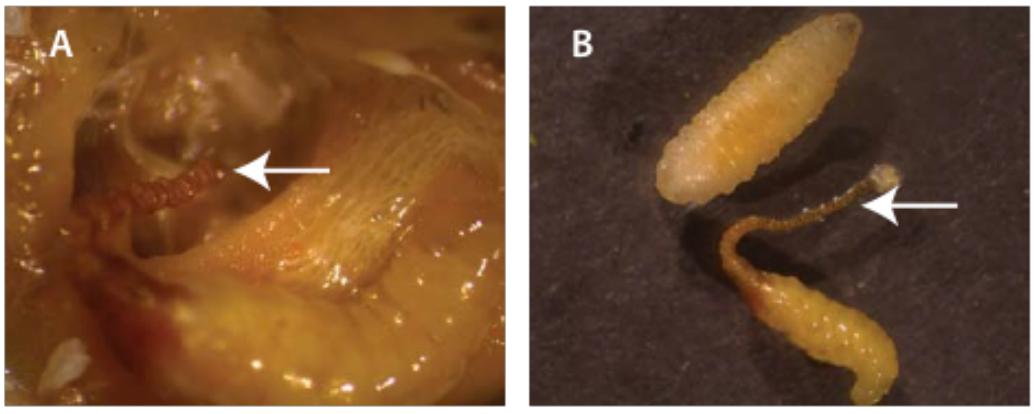


Figure A.2: *Blaesoxphia* larval development.
A. Larvae reside in the abdominal cavity of the grasshopper were they consume the fat body, ovaries or testis during development. The respiratory tube leads to the cuticle. (exterior view Fig. 7.1.) B. Mature larvae after emergence (top) and the dissected developing larva with respiratory tube attached.

posterior end of the grasshopper emerging through the genitals (Fig. A.3). This results in the death of the host.

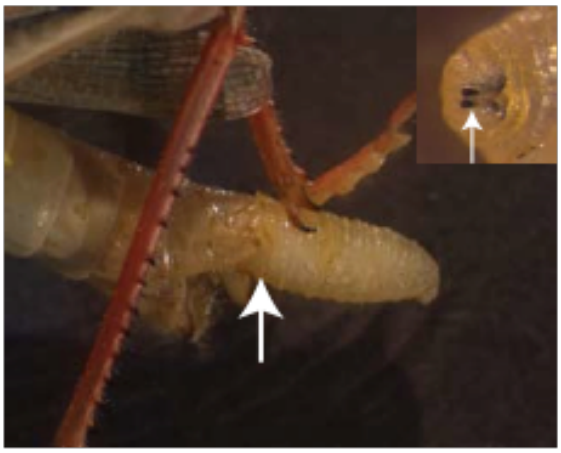


Figure A.3: Posterior *Blaesoxphia* larval exit.
The larvae exit the grasshopper from the posterior. Inset: Mature larvae possess a set of hooked mouthparts used to escape from the grasshopper host. These mouthparts are absent or undeveloped in the immature larvae.

Metamorphosis follows the emergence from the grasshopper within 24 hours (personal observation). Autopsies of grasshoppers following larval emergence revealed a completely parasitized abdomen (Fig A.4).

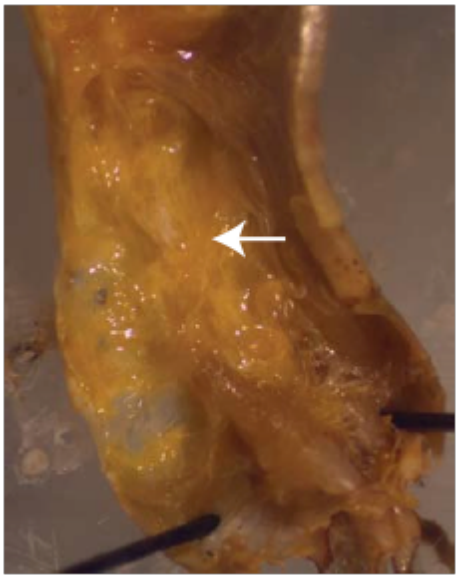


Figure A.4: Abdominal parasitism of host fat body and ovary.
Gross dissection of female grasshopper following larval emergence revealed a parasitized abdominal cavity. Fat body and ovaries are no longer present.

During field collections of populations experiencing outbreak conditions the density of *Blaesoxiphas* can be quite high. The unintentional capture of flies during sweep netting is important to note. Flies that remain inside group cages with field-caught grasshoppers can deposit larvae if not culled as soon as they are caught. A simple method for accomplishing this is to extend the sock netting of the cage inside and trap the fly against the wall of the cage killing the fly. It is especially useful to hold the cage against the prevailing sunlight to silhouette the flies against the screen of the cage. During out break conditions in Arizona as many as 5 flies were caught for every 50 grasshoppers (Personal observation).

Microsporidian Parasites

Microsporidians represent the largest single threat to the successful rearing of a colony of *M. sanguinipes*, especially one that requires introduction of new individuals annually from field collections for its maintenance. Microsporidians are obligate intercellular parasites of eukaryotes (Larsson, 2004). They seem to be more related to fungi than animals (Larsson, 2004). They are characterized by a life cycle that includes a highly resilient spore coat, a coiled filament employed to burrow between adjacent tissues and transmission that typically involves ingestion of the spore and infiltration of the digestive lining (Agnew et al., 2003). During grasshopper migrations and outbreaks the high population density and the canabilistic nature of *M. sanguinipes* provide ideal conditions for the rapid proliferation of microsporidiosis among the population. This is especially important for the researcher performing field collections as the removal of dead or dying individuals from collection cages can greatly reduce the incidence of microsporidian infection in the F1 generation (Personal observation). The two major microsporidian parasites of *Melanoplus* are *Maloemobae locustae* and *Nosema locustae* . *Maloemobae* can be found in low numbers in the malpigian tubules of many healthy field grasshoppers (Personal observation). It is possible that maloemeba sits on the border between symbiote and pathogen since it is almost always present but infrequently progresses to pathogenesis (Agnew et al., 2003). The pathology of maloemeba is generally characterized by listlessness, sudden dark coloration and finally liquefaction of the body cavity (personal observation). The carcass at this stage is composed primarily of spores

and as such is extremely infectious. Removal of carcasses and aseptic technique are key. *Nosema locustae* is important not so much for its ecological role in *Melanoplus* populations but for its continued role as an introduced bio-control (Henry and Oma, 1981). The symptoms of *Nosema* infection are different from those of maloemeba. The pathology is primarily in the gut and reproductive tract. Red scab-like structures form on the external genitalia of individuals infected with Nosema (Fig. 7.5). Grasshoppers presenting these symptoms are like those of individuals infected with *Maoloemeba* highly infectious and should be treated with care during disposal. *Nosema* baits have been used by land owners and agricultural agencies for the control of outbreak populations of grasshoppers over the past 40 years (Henry, 1977).

Figure A.5: Symptoms of microsporidian infection in *M. sanguinipes*.
Fifth instar nymphs of *M. sanguinipes* infected with *Nosema locustae*. A. Lethargic but living individual presenting early genital symptoms of infection. B & C. Recently deceased individuals heavily infected with Nosema. B is also showing symptoms of a concurrent *Malaomeba* infection. Note dark abdominal coloration.

Nosema spores have been shown to persist for years in soils and it is highly advisable to perform diagnostic cell smears for the presence of either the characteristic vegetative stage (xenoma Fig. A.6.c) of the pathogen or the

presence of the spores (Fig. A.6.a). The spores are visible under phase contrast microscopy with or without staining (Fig. A.6.b).

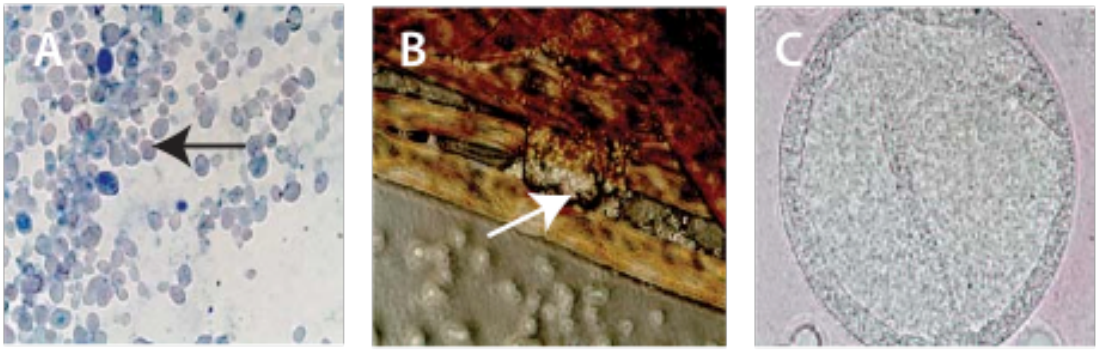


Figure A.6: Micrographs of microsporidians.
a. Phase contrast (1000x) of giemsa stained spores of *Nosema locustae* and *Maloemeba locustae*. b. *Malemeba locustae* spores in the malpigian tubules of *M. sanguinipes*. c. Phase contrast (400x) image of xenoma characteristic of early microsporidiosis in *M. sanguinipes* fat body.

Methods:

Microscopy

Stereomicroscope images (Fig. 6.1 - 6.5) were collected on a Wild TYP 355110 microscope with a Leica DFC280 digital camera using Leica FireCam Version 1.7.1 (Leica, UK). Bright field and phase contrast stage microscopy images were collected using an Olympus CX31 microscope.

Cell smears and Giemsa staining

Tissue smears were performed following methods of Poinar and Thomas (1984). Tissue was dissected and smeared on a clean glass slide and allowed to air dry then fixed in 100% methanol for 5 minutes and again air dried. Fixed

slides were stained with a 1:1000 dilution of Giemsa (Sigma-Aldrich), washed in distilled water and air dried. Tissues were examined immediately or mounted for storage.

Discussion

The parasites and pathogens discussed here illustrate the dangerous environment that *M. sanguinipes* inhabits. The microsporidian parasites are primarily a concern for the researcher in the context of maintaining a healthy laboratory colony. In the field they appear to be a selective pressure only in out-break populations where the high density and frequency of cannibalism can create situations where the pathogens can flourish. The proximity of anthropogenic hazards such as highways can also create a higher degree of mortality in dense populations and opportunities for cannibalism. The long term impact of repeated anthropogenic introduction of microsporidian pathogens remains to be seen. How these hardy and opportunistic pathogens alter the ecology and population biology of grasshoppers will be an important question in the coming years.

The *Blaesoxipha* flies have existed with the *Melanoplus* genus for a long period of time. As a selective pressure on the immune system of *Melanoplines* in the context of flight it seems obvious that grasshoppers performing flights would have a selective advantage if they evolved up-regulation of a system that powers flight, acts in immune surveillance and is part of the first immune response.

Whether increased lipophorin titers might afford additional protection against parasitic flies remains to be investigated, but it is clear that selective pressures against the immunity-flight tradeoff exist.

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